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(54) Title: PROCESS FOR EX VIVO EXPANSION OF HEMATOPOIETIC PROGENITOR CELLS

(57) Abstract

The present inention relates to a process for *ex vivo* expansion of hematopoietic progenitor cells. This process is particularly useful in conjunction with a process for conducting autologous hematopoietic progenitor cell transplantation. In this process, hematopoietic progenitor cells are obtained from a patient prior to cytoreductive therapy. The hematopoietic progenitor cells are then expanded *ex vivo* with bone marrow endothelial cells or with one or more cytokines therefrom to produce a cellular preparation with an increased number of hematopoietic progenitor cells. The cellular preparation is then administered to the patient in conjunction with or after cytoreductive therapy.

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PROCESS FOR *EX VIVO* EXPANSION OF HEMATOPOIETIC PROGENITOR CELLS

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FIELD OF THE INVENTION

The present invention relates to a process for ex vivo expansion of hematopoietic progenitor cells with human bone marrow endothelial cells or cytokines derived therefrom.

BACKGROUND OF THE INVENTION

1. The Bone Marrow Environment

The bone marrow microenvironment is a complex, three dimensional structure where hematopoietic elements proliferate, differentiate, mature, and ultimately migrate into the circulation as mature erythrocytes, lymphocytes, granulocytes, monocytes, and platelets. Stromal cells, which form the backbone of the bone marrow microenvironment, consist of fibroblasts, endothelial cells, adipocytes, osteoclasts, and monocytes. They secrete cytokines, produce extracellular matrix, and mediate direct cellular contact which regulates hematopoiesis. The fibroblasts of the bone marrow adventitia are composed of adventitial reticular cells ("ARC")(Aizawa, S., et al., "Molecular Basis of the Recognition of Intravenously Transplanted Hemopoietic Cells by Bone Marrow," Proc. Natl. Acad. Sci. USA, 85:3180 (1988), which is hereby incorporated by reference), perisinusoidal adventitial cell ("PAS"), periarterial adventitial cells

("PAA"), and intersinusoidal reticular cells ("ISR") (Tavassoli, M., et al., "Homing Receptors for Hemopoietic Stem Cells are Lectins with Galacyosyl and Mannosys Specificities," Trans. Assoc. Am. Phys., 100:294 (1994), which is hereby incorporated by reference). Most studies on stromal regulation of hematopoiesis derive from investigations of bone marrow fibroblasts. However, little is known about the role of bone marrow derived microvascular endothelial cells ("BMEC") in the regulation of hematopoiesis. The anatomic location of bone marrow microvascular endothelium suggests that it may serve as a gatekeeper regulating the passage of cells between the marrow and the circulation (Aizawa, S., et al., "Molecular Basis of the Recognition of Intravenously Transplanted Hemopoietic Cells by Bone Marrow," Proc. Natl. Acad. Sci. USA, 85:3180 (1988); Tavassoli, M., et al., "Homing Receptors for Hemopoietic Stem Cells are Lectins with Galacyosyl and Mannosys Specificities," Trans. Assoc. Am. Phys., 100:294 (1994); Tavassoli, M., "Localization of Megakaryocytes in the Bone Marrow," Blood Cells, 15:3 (1989); and Springer, T.A., "Adhesion Receptors of the Immune System," Nature, 346:425 (1990), which are hereby incorporated by reference), but its role in regulating the trafficking and development of hematopoietic cells has not been well studied because of the lack of methodologies to isolate and grow BMEC. Shimuzu, Y., et al., "Lymphocyte Interactions with Endothelial Cells," Immunology Today, 13:106 (1992) and Zimmerman, G.A., et al., "Endothelial Cell Interactions with Granulocytes: Tethering and Signaling Molecules," Immunology Today, 13:93 (1992), which are hereby incorporated by reference.

Studies on other endothelial cell types, particularly human umbilical endothelial cells ("HUVEC"), have shown that the trafficking of immune or inflammatory cells across the endothelium is regulated by the coordinate

surface expression of receptors specific for a subset of circulating cells. Hynes, R.O., "Integrins: Versatility, Modulation, and Signaling in Cell Adhesion," Cell, 69:11 (1992); Tavassoli, M., et al., "Molecular Basis of Homing Intravenously Transplanted Cells to the Marrow," Blood, 76:1059 (1990); Hardy, C.L., "Distribution of Homing Protein on Hemopoietic Stromal and Progenitor Cells," Exp. Hematol., 19:968 (1991); and Teixido, J., et al., "Role of B1 and B2 Integrins in the Adhesion of Human CD34 Stem Cells to Bone Marrow Stroma," J. Clin. Invest., 90:358 (1992), which are hereby incorporated by reference). Several of these surface receptors are induced by inflammatory cytokines such as interleukin-1β (IL-1β). Tavassoli, M., et al., "Molecular Basis of Homing Intravenously Transplanted Cells to the Marrow," Blood, 76:1059 (1990), which is hereby incorporated by reference. Similarly, the specific expression of unique adhesion molecules on the surface of bone marrow microvascular endothelium may regulate the trafficking of hematopoietic elements, particularly pluripotent stem cells in and out of the bone marrow microenvironment. Simmons, P.J., et al., "Vascular Cell Adhesion Molecule-1 Expressed by Bone Marrow Stromal Cells Mediate the Binding of Hematopoietic Progenitor Cells," Blood, 80:388 (1992); Long, M.W., et al., "Thrombospondin Functions as a Cytoadhesion Molecule for Human Hematopoietic Progenitor Cells," Blood, 75:2311 (1990); Koenigsmann, M., et al., "Myeloid and Erythroid Progenitor Cells from Normal Bone Marrow Adhere to Collagen Type I," Blood, 79:657 (1992); Campbell, A., et al., "Hemonectin: A Novel Hematopoietic Adhesion Molecule," Prog. Clin. Biol. Res., 352:97 (1990); Gordon, M.Y., et al., "Heparin Sulfate is Necessary for Adhesive Interactions Between Human Early Hemopoietic Progenitor Cells and the Extracellular Matrix of the Marrow Microenvironment," Leukemia, 2:804 (1988); Long, M.W., et al., "Human Hematopoietic Stem Cell Adherence to Cytokine and Matrix

Molecules," J. Clin. Invest., 90:251 (1992); Lewinsohn, D.M., et al., "Hematopoietic Progenitor Cell Expression of the H-CAM (CD44) Homing-Associated Adhesion Molecule," Blood, 75:589 (1990); Arkin, S.B., et al., "Expression of Intercellular Adhesion-1 (CD54) on Hematopoietic Progenitors," Blood, 77:948 (1991); QixkeMainghe, S.N., "Observations on the Ultrastructure of Sinusoids and Reticular Cells in Human Bone Marrow," Clin. Lab. Haematol., 13:263 (1991); Watanabe, Y., "Fine Structure of Bone Marrow Stroma," Acta. Haematol. Jpn., 48:1688 (1985); Sieff, C.A., "The Production of Hematopoietic Growth Factors by Endothelial Accessory Cells," Blood Cells, 13:65 (1987); Segal, G.M., et al., "Erythroid Burst Promoting Activity Produced by Interleukin-1 Stimulated Endothelial Cells is Granulocyte-Macrophage-Colony Stimulating Factor," Blood, 72:1364 (1988); and Broudy, V.C., et al., "Interleukin 1 Stimulates Human Endothelial Cells to Produce Granulocyte-Macrophage Colony Stimulating Factor and Granulocyte Colony Stimulating Factor," J. Immunol., 139:464 (1987), which are hereby incorporated by reference.

Within the bone marrow microenvironment, BMEC reside in close association with other cell types such as fibroblasts, adipocytes, mature megakaryocytes, plasma cells, and hematopoietic cells, and form an interface between the circulation and the hematopoietic compartment. Aizawa, S., et al., "Molecular Basis of the Recognition of Intravenously Transplanted Hemopoietic Cells by Bone Marrow," Proc. Natl. Acad. Sci. USA, 85:3180 (1988); Tavassoli, M., et al., "Homing Receptors for Hemopoietic Stem Cells are Lectins with Galacyosyl and Mannosys Specificities," Trans. Assoc. Am. Phys., 100:294 (1994); Tavassoli, M., "Localization of Megakaryocytes in the Bone Marrow," Blood Cells, 15:3 (1989); and Springer, T.A., "Adhesion Receptors of the Immune System," Nature, 346:425 (1990), which are hereby incorporated by reference. This

anatomy suggests that BMEC, through direct cellular contact, may control the final stages of hematopoiesis, regulating the entry of mature cells into the peripheral circulation. The release of cytokines by the bone marrow endothelium, independent of cell contact, may also have a role in the regulation of hematopoiesis. Bagby, G.C., et al., "Vascular Endothelial Cells and Hematopoiesis: Regulation of Gene Expression in Human Vascular Endothelial Cells," Hematologic Pathology, 5:93 (1991); Hart, M.N., et al., "Brain Microvascular Smooth Muscle and Endothelial Cells Produce Granulocyte Macrophage Colony-Stimulating Factor and Support Colony Formation of Granulocyte-Macrophage-Like Cells," Am. J. Path., 421:1141 (1992); Baumhueter, S., et al., "Binding of L-Selectin to the Vascular Sialomucin CD34," Science, 262:436 (1993); Terstappen, L.W., et al., "Sequential Generation of Hematopoietic Colonies Derived from Single Nonlineage Committed CD34+CD38- Progenitor Cells," Blood, 77:1218 (1991); and Gunji, Y., et al. "Expression and Function of Adhesion Molecules on Hematopoietic Stem Cells: CD34+ LFA1- Cells are More Primitive than CD34+ LFA1+ Cells," Blood, 80:429 (1992), which are hereby incorporated by reference.

2. Cancer Therapy

Cancers are generally treated with various forms of cytoreductive therapies. Cytoreductive therapies involve administration of ionizing radiation or chemical toxins which are cytotoxic for rapidly dividing cells. Side effects of such therapy can be attributed to cytotoxic effects upon normal cells and can usually limit the use of cytoreductive therapies. A frequent side effect is myelosuppression, or damage to bone marrow cells which gives rise to white and red blood cells and platelets. Myelosuppression causes patients to develop cytopenia and,

as a result, an increased risk of infection and bleeding disorders.

Cytopenia is a major factor contributing to morbidity, mortality, and under-dosing in cancer treatment. Many clinical investigators have manipulated cytoreductive therapy dosing regimens and schedules to increase dosing for cancer therapy, while limiting damage to bone marrow. One approach involves bone marrow transplantations in which bone marrow hematopoietic progenitor cells are removed before a cytoreductive therapy and then reinfused following therapy to rescue bone marrow from toxicity resulting from the cytoreductive therapy. Progenitor cells can then implant in bone marrow and differentiate into mature blood cells to supplement reduced population of mature blood cells.

High-dose chemotherapy is therapeutically beneficial because it produces an increased frequency of objective response in patients with metastatic cancers, particularly breast cancer, compared to standard dose therapy. This can result in extended disease-free remission for even poor-prognosis patients. Nevertheless, high-dose chemotherapy is toxic and many resulting clinical complications are related to infections, bleeding disorders and other effects associated with prolonged periods of myelosuppression.

Currently, a human recombinant granulocyte macrophage-colony stimulating factor ("GM-CSF") analog protein (i.e. sargramostim) is available in the U.S. for accelerating hematopoietic recovery following bone marrow transplantations. Sargramostim treatment has reduced many complications associated with bone marrow transplantations.

The existence of both marrow borne and circulating hematopoietic stem cells has been demonstrated using a variety of experimental studies and cell culture techniques. Two colony stimulating factors, GM-CSF and granulocyte colony stimulating factor ("G-CSF"), have been shown to

increase the frequency of circulating hematopoietic progenitor or stem cells. Several studies (Gianni, et al., Lancer, 335:589 (1989); Siena, et al., Blood 74:1905 (1989); and Molineux, et al., Blood 76:2153 (1990)) describe *in vivo* administration of GM-CSF to increase the transplantation potential and frequency of primitive progenitor cells in a population of peripheral blood cells obtained from patients with tumors. These procedures represent attempts to rescue chemotherapy-induced suppression of bone marrow by administering GM-CSF *in vivo* to recruit bone marrow progenitor cells into peripheral blood and then later administering harvested hematopoietic progenitor cells to patients.

More specifically, Gianni, et al., describe a clinical study in which patients received high dose cyclophosphamide (i.e. 7 g/m^2) and were transplanted with autologous peripheral blood progenitor cells and autologous bone marrow cells. Patients who were treated with GM-CSF as a progenitor cell recruitment agent, prior to harvesting peripheral blood progenitor cells, recovered more quickly from cytopenia than patients whose peripheral blood progenitor cells were not recruited by GM-CSF. Thus, GM-CSF administration increased the number of peripheral blood progenitor cells. This protocol resulted in more rapid hematopoietic recovery in tested patients than in control patients who received chemotherapy without autologous bone marrow transplantation but with peripheral blood progenitor cell support.

Cancer patients treated with high doses of chemotherapy and autologous bone marrow transplantation, who received subsequent GM-CSF treatment, have shown faster myeloid recovery than similarly treated historical controls (Brandt, et al., N. Engl. J. Med., 318:869 (1988) and Nemunatis, et al., Blood, 72:834 (1988)). Studies have shown that the time to achieve a minimum granulocyte count

of 0.5 x 10^9/1 after cytoreductive therapy was shorter in patients receiving GM-CSF. Granulocyte count increases were most pronounced during GM-CSF infusion. After discontinuation of GM-CSF, leukocyte counts in treated patients fell to control levels (Brandt, et al., supra).

GM-CSF is also useful for autologous bone marrow transplantation following cytoreductive therapy. Socinski, et al., Lancet, 331:194 (1988) reported that GM-CSF administration after cytotoxic chemotherapy expands a circulating pool of hematopoietic progenitor cells by approximately 60-fold. Others have reported that human mononuclear cells circulating in the circulating blood, particularly during recovery from chemotherapy-induced myelosuppression, have been used successfully to reconstitute hematopoiesis after fully myeloablative (complete bone marrow toxicity) treatments (See, e.g., Bell et al., Hematol. Oncol., 5:45 (1987)).

Mason, et al., Proc. Amer. Assoc. Cancer Res., 32:193 (1991), reported that in vitro interleukin-3 (IL-3) alone or in combination with interleukin-6 (IL-6) increased the number of colony forming progenitors from human blood progenitor cells two fold in vitro. Mason et al. also reported that GM-CSF did not expand the colony forming progenitor population in vitro.

U.S. Patent No. 5,199,942 to Gillis relates to a method for improving autologous transplantation by expanding hematopoietic progenitor cells ex vivo with an ex vivo growth factor selected from the group consisting of interleukin-3, steel factor, granulocyte macrophage-colony stimulating factor, interleukin-1, granulocyte macrophage-colony stimulating factor/interleukin-3 fusion proteins, and combinations thereof.

Improvements in autologous hematopoietic cell transplantation are needed further to speed recovery from cytoreductive therapy and to allow for the use of higher and

more effective doses of such therapies. The present invention provides such an improvement.

SUMMARY OF THE INVENTION

The present invention relates to a process for ex vivo expansion of hematopoietic progenitor cells. In accordance with this process, hematopoietic progenitor cells are provided and expanded ex vivo with bone marrow endothelial cells or with one or more cytokines from those cells. As a result, a cellular preparation with an increased number of hematopoietic progenitor cells is produced.

This process is particularly useful in conjunction with a process for conducting autologous hematopoietic progenitor cell transplantation. In this process, hematopoietic progenitor cells are obtained from a patient prior to cytoreductive therapy. The hematopoietic progenitor cells are then expanded ex vivo with bone marrow endothelial cells or with one or more cytokines therefrom to produce a cellular preparation with an increased number of hematopoietic progenitor cells. The cellular preparation is then administered to the patient in conjunction with or after cytoreductive therapy.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is an immunofluorescence from an intact bone marrow spicule stained with monoclonal antibody to factor VIII/vWF demonstrating selective staining of endothelial cells and megakaryocytes (arrows). Note the complex vascular network, crisscrossing the spicule. Large polyploid megakaryocytes can be seen in association with endothelial cells (100X).

Figure 1B is a photograph of partially collagenase (0.1%) digested (10 minutes) bone marrow spicule, retained on a 40 micron mesh, washed three times with buffer A, and transferred to a plastic slide and stained with Wright/Giemsa stain. This photograph demonstrates the complex network of microvasculature within a typical bone marrow spicule. Note that the spicule is denuded of hematopoietic elements and has a predominant central capillary (large arrow) branching into single layered sinusoidal endothelial cells (small arrows) (50X).

Figure 2A is a phase contrast micrograph (100x) of a typical microvessel isolated from bone marrow aspirate. Bone marrow spicules retained on a 40 micron mesh were digested with 0.1% collagenase for 30 minutes at 37°C, and pushed through a 21 gauge needle. The digested material was passed through another 40 micron mesh, and retained microvessel fragments were resuspended in ECGM and transferred to a 24 well cluster plate. Note that the microvessel is tightly attached to the gelatin coated plastic dish.

Figure 2B is an immunohistochemistry showing factor VIII/vWF staining of a microvessel using monoclonal anti-factor VIII/vWF antibody and immunoperoxidase detection (100X). Note the spindle shaped endothelial cell lining the microvessel. Red stain is peroxidase substrate, amino-ethyl carbazole.

Figure 2C shows colonies of BMEC emerging from attached microvessels after five days of incubation in ECGM (50X).

Figure 2D shows monoclonal anti-CD34 (HPCA-1, BD) staining with immunoperoxidase detection of a typical microvessel, demonstrating the strong expression of this antigen throughout the microvessel. Red stain is alkaline phosphatase substrate, fast red (100X).

Figure 3A is a phase contrast microscopy of a monolayer of BMEC grown from a microvessel explant and purified after Ulex selection. Note the spindle-like and cobblestone morphology of these endothelial cells (50X).

Figure 3B is an epifluorescence of similar monolayer demonstrating that greater than 98% of cells show fluorescence, characteristic of acetylated-LDL uptake (50X).

Figure 3C is an immunoperoxidase staining with monoclonal antibody to factor VIII/vWF of BMEC monolayers (50X).

Figure 3D is an immunoperoxidase staining with monoclonal antibody to CD34 antigen (HPCA-1). Only early passage cells express this antigen (120X).

Figure 3E is an immunoperoxidase stain with monoclonal antibody to PECAM, demonstrating the expression of this antigen specifically at cellular junctions (50X).

Figure 3F is a negative control, immunoperoxidase stain with monoclonal mouse IgG. Red stain is peroxidase substrate amino-ethyl carbazole (50X).

Figure 4A is an electron microscopic analysis of a BMEC monolayer showing a typical endothelial cell with numerous mitochondria and Weibel-Palade bodies.

Figure 4B is a higher magnification of the same BMEC demonstrating the presence of cigar shaped Weibel palade bodies (arrows) which is characteristic of vascular endothelium.

Figure 5 shows CD34+ progenitor cell adhesion to BMEC. Bone marrow derived CD34+ cells isolated by immunoadsorption technique, were incubated with BMEC monolayers at 37°C, in the presence or absence of blocking antibodies for one hour and adherent cells were quantified by phase contrast microscopy.

Figure 6 shows CD34+ progenitor cell adhesion to IL-1β stimulated BMEC monolayers. BMEC monolayers were incubated with IL-1β (10 ng/ml) for 16 hours, then incubated

or with one or more cytokines from those cells. As a result, a cellular preparation with an increased number of hematopoietic progenitor cells is produced.

In its more preferred form, the present invention relates to a process for conducting autologous hematopoietic progenitor cell transplantation. In this process, hematopoietic progenitor cells are obtained from a patient prior to cytoreductive therapy. These cells are expanded ex vivo with bone marrow endothelial cells or with one or more cytokines from those cells to produce a cellular preparation with an increased number of hematopoietic progenitor cells. The cellular preparation is then administered to the patient in conjunction with or after cytoreductive therapy.

Progenitor cells may be obtained from human mononuclear cells produced by bone marrow and peripheral blood. Progenitor cells may be separated from peripheral blood, for example, by density gradient centrifugation such as with a Ficoll Hypaque® system. Another means for separating hematopoietic progenitor cells obtained from bone marrow or peripheral blood involves separating with antibodies that recognize a stage-specific antigen on immature human hematopoietic progenitor cells. One example of an antibody recognition method for separating human hematopoietic progenitor cells is described in U.S. Patent No. 5,035,994 to Civin, which is incorporated by reference.

Once hematopoietic progenitor cells are obtained, they may be stored in cryogenic conditions or expanded ex vivo according to the present invention. Stored cells may later be rapidly thawed and expanded ex vivo according to the present invention.

Ex vivo use of bone marrow endothelial cells or cytokines therefrom is by direct addition to cultures of hematopoietic progenitor cells in physiological buffer or culture medium. Preferred progenitor cells expansion medium is, for example, minimal essential medium supplemented with

autologous serum and antibiotics. Progenitor cell expansion media, according to the present invention, comprises autologous serum and possibly antibiotics. Other culture media include for example, Hanks, McCoys, RPMI 1640 minimal essential media (MEM) and others, and include from 1% to 20% autologous serum and possibly antibiotics. Cells are cultured ex vivo in the presence of bone marrow endothelial cells or cytokines therefrom for at least one day and no more than 2 weeks.

The bone marrow endothelial cells can be characterized in a number of different ways, including by ATCC Accession No. CRL11666, submitted June 20, 1994. A cell line can be produced by transforming the bone marrow endothelial cells using conventional procedures. B. Schwartz, et al., "Mammalian Cell Lines Can Be Efficiently Established *In Vitro* Upon Expression of the SV40 Large T Antigen Driven By A Promoter Sequence Derived From The Human Vimentin Gene," Biol. Cell, 73:7-14 (1991), which is hereby incorporated by reference.

One characteristic of the bone marrow endothelial cells of the present invention is that they support adhesion of megakaryocytes and bone marrow mononuclear cells. The bone marrow mononuclear cells can be CD34+progenitor cells, plasma cells, erythroid cells, and myeloid cells. The megakaryocytes and CD34+progenitor cells bind to the bone marrow endothelial cells of the present invention at a level of 20 to 30 times greater than the amount of megakaryocytes and CD34+progenitor cells in bone marrow in the presence of divalent cations. Binding the CD34+progenitor cells to the bone marrow endothelial cells is inhibited by CD34 antibodies and ethylenediaminetetraacetic acid. Binding of the CD34+progenitor cells to the bone marrow endothelial cells is enhanced by treatment of these cells with interleukin-1β with such binding being divalent cation independent and not inhibited by ethylenediaminetetraacetic

acid or CD34 antibodies. The bone marrow endothelial cells show selective adhesion of CD34+HLA-DR$^-$ CD38$^-$phenotype cells.

The bone marrow endothelial cells are a source for one or more cytokines supporting progenitor cell proliferation. The bone marrow endothelial cells have an enhanced ability to cause progenitor cell proliferation compared to bone marrow fibroblasts and human umbilical cord vein endothelial cells. Such one or more cytokines support CD34+ pluripotent progenitor cell self-renewal. These cytokines can be used in the process of the present invention instead of the cells per se.

The bone marrow endothelial cells are also a source for one or more cytokines supporting differentiation of pluripotent progenitor cells to megakaryocytes. These cytokines can be used in the process of the present invention instead of the cells per se. As explained previously, pluripotent cells differentiate ultimately to form a number of different mature blood cells. When this occurs, such progenitor cells must be replenished to continue the production of new mature blood cells. As demonstrated *infra* in the examples, the bone marrow endothelial cells produce one or more cytokines which support the renewal of such pluripotent cells. The results obtained demonstrate that the normal depletion of stem cells as measured by the ability to form colonies in semi-solid agar assays is markedly delayed by post-culture supernatant from bone marrow endothelial cells. No known combination of cytokines produces similar activity.

The process of the present invention can also utilize one or more cytokines supporting platelet formation by megakaryocytes instead of the cells. The initial stage of megakaryocyte development involves sequential proliferation of CD34+pluripotent stem cells into megakaryocyte blast forming units ("BFU-MK"), and colony forming units ("CFU-MK"), which eventually mature into

megakaryoblast ("MK-blast"). Only 0.5 to 2% of CD34+progenitor cells and 10 to 30% of CFR-ML stain positively for GPIIb/IIIa. Cytokines such as interleukin-3 ("IL-3") and granulocyte macrophage-colony stimulating factor ("GM-CSF") are believed to regulate the proliferation of these early megakaryocytic precursors. A lineage-specific promoter of megakaryocytic maturation ("MSF") that may specifically commit CD34+progenitor cells to a megakaryocytic lineage has been hypothesized. Later megakaryocytic development is characterized by nuclear endoreplication of precursors with a recognizable megakaryocytic phenotype and the acquisition of additional phenotypic characteristics of mature megakaryocytes. As demonstrated *infra* in the examples, the bone marrow endothelial cells of the present invention produce one or more cytokines which support the differentiation of pluripotent progenitor cells to megakaryocytes. Although several cytokines can support megakaryocyte differentiation from pluripotent stem cells (i.e. IL3, IL6, GM-CSF), none alone or in combination result in the percentage of megakaryocytic lineage commitment observed with bone marrow endothelial cells or bone marrow endothelial cells post culture supernatant.

For example, the close association of megakaryocytes and bone marrow endothelial cells has been noted and is responsible for the daily production of 2×10^{11} platelets. The platelets are formed within the megakaryocytes and then released into circulation through the bone marrow endothelial cells. This is achieved as a result of the megakaryocytes extending pseudopodia through the bone marrow endothelial walls which are cut off to release the platelets. As demonstrated *infra* in the examples, the isolated bone marrow endothelial cells produce one or more cytokines which support platelet formation from mature megakaryocytes.

The above-described cytokines can be obtained from a cDNA expression library prepared from poly(A)+RNA derived from early passage bone marrow endothelial cell monolayers. This is described in more detail in the examples.

The isolated bone marrow endothelial cells produce a number of known cytokines, including interleukin-3, interleukin-6, granulocyte-colony stimulating factor, kit ligand, and granulocyte macrophage colony stimulating factor. As demonstrated in the examples, these cytokines are elaborated by the bone marrow endothelial cells at the following levels: 32 ± 11 pg/ml for interleukin-3, 292 ± 21 pg/ml for interleukin-6, 3000 ± 478 pg/ml for kit ligand, 345 ± 32 pg/ml for granulocyte-colony stimulating factor, and 373 ± 36 pg/ml for granulocyte macrophage colony stimulating factor.

In essence, the bone marrow endothelial cells or cytokines therefrom are capable of supporting hematopoietic cells in much the same fashion as endothelial cells in bone marrow do. It is at the medullary marrow spaces where the endothelial cells line microvessels and sinusoids that post-embryonic hematopoiesis is localized. During this process, progenitor cells traffick to and from this site where they bind and begin proliferating and differentiating into various mature hematopoietic cells.

The bone marrow endothelial cells are isolated by a process which initially involves an aspiration process, as described, for example, in M.M. Wintrobe, et al., Clinical Hematology, pp. 59-61 (8th ed. 1981), which is hereby incorporated by reference. This aspirate must be a vigorously-obtained, deep bone marrow aspirate in order to obtain a high yield of spicules containing vessel fragments. Such a product is obtained by vigorously repositioning the bone marrow needle during aspiration from deep within the bone marrow cavity. Bone marrow spicules are then recovered from the bone marrow and digested with proteolytic enzymes.

Microvessel fragments from the digested spicules are then recovered, and these fragments are grown as explants to form the bone marrow endothelial cells of the present invention.

Filters can be used in both the steps of recovering the bone marrow spicules from bone marrow and of recovering microvessel fragments from the digested spicules. In both cases, the desired material (i.e., the spicules or the microvessel fragments) are retained on the filter. The spicules and the microvessel fragments can be recovered with a filter made from stainless steel or polypropylene which has a 40-200 micron mesh.

Once recovered, these spicules can be resuspended in a buffer for subsequent digestion. Suitable buffers include saline-type buffers with ethylenediaminetetraacetic acid ("EDTA") added, such as Hanks Buffered Saline with EDTA.

Digestion of spicules with one or more proteolytic enzymes is carried out at about 30-40°C. The proteolytic enzyme is used in a concentration of 0.05 to 0.5% and, preferably, is collagenase. Trpysin is another potentially useful proteolytic enzyme.

Microvessel fragments recovered from the digested spicules are grown as explants in endothelial cell growth medium. To do this, the microvessels are washed and then collected by gentle vortexing or trituration. The material collected is plated on fibronectin or gelatin coated plastic dishes. After 5-7 days of growth, a mixed population of BMEC and other adventitial cells are present.

Once bone marrow endothelial cells are grown from the microvessel fragments, these cells should be separated from contaminating fibroblasts and adipocytes. One technique of achieving such separation involves binding the bone marrow endothelial cells to magnetic particles coated with a material to which these cells bind, and separating

magnetically the magnetic particles with surface bound bone marrow endothelial cells from other materials.

Hematopoietic progenitor cells, treated ex vivo in accordance with the process of the present invention, are readministered to patients by autologous transplantation. Generally, this occurs after the hematopoietic progenitor cells are cultured (i.e. expanded) ex vivo in the presence of bone marrow endothelial cells or cytokines therefrom for 1 to 14 days. The expanded cells can be stored after expansion under cryogenic conditions before administration to the patient. After washing, the expanded cells are administered within 72 hours of cytoreductive therapy. Hematopoietic progenitor cell administration is usually carried out by infusion over several days. Preferably, 10^7 to 10^9 of expanded mononuclear cells per kilogram are administered to the patient for an autologous transplantation.

EXAMPLES

Example 1 - Isolation of Endothelial Cells from Bone Marrow Aspirate

Bone marrow aspirates were obtained with a standard Jamshidi needle in preservative free heparin (50 units/ml) from posterior or anterior iliac crests of normal volunteer donors undergoing bone marrow harvest at Memorial Sloan-Kettering Cancer Center (MSKCC). Each 3 ml of bone marrow aspirate obtained from a single bone marrow puncture resulted in removal of approximately 150 to 300 floating spicules with sizes ranging from 250 to 500. All steps of isolation were performed at room temperature. If the bone marrow aspirate was stored at 4°C, it was allowed to warm up to room temperature before processing to avoid solidification of fatty components which interfered with filtration steps. Since bone marrow spicules adhere avidly

to plastic or glass surfaces, all glassware or plastic pipets used in these experiments were passivated with 1% bovine serum albumin ("BSA") to avoid attachment and loss of spicules.

The bone marrow aspirate, consisting of floating fat laden spicules, was immediately diluted 1:1 in Hanks balanced salt solution ("HBSS") with 1mM EDTA (i.e., buffer A), and passed through a 40 micron stainless steel filter to remove the loosely attached hematopoietic cells. The retained stromal elements, which consisted mostly of fat laden spicules, were washed with 50 ml of buffer A to remove loosely attached cells and plasma. The filter was then placed in a 50 ml conical tube and the retained spicules were resuspended in 5 ml of buffer A, followed by the addition of 5 ml of 0.2% collagenase (final concentration of 0.1%) for 20 to 30 minutes at 37°C. Occasionally, a sample of the material undergoing collagenase digestion was removed and examined by phase contrast microscopy to assess the adequacy of digestion. The digested material was passed gently through a 20 or 21 gauge needle, and then refiltered through another 40 micron filter to obtain microvessel fragments The retained microvessels were washed with 30 ml of buffer A, and then collected by gentle vortexing or trituration of the filters in a 50 ml conical tube and plated on fibronectin or gelatin coated 12 or 6 well cluster plastic dishes. Centrifugation of the microvessels for two minutes at 150g accelerated their attachment to the plastic dish.

After 5 to 7 days of growth of microvessel explants in endothelial cell growth medium ("ECGM") containing M199 medium (MA, Bioproducts), heparin 90 μg/ml (Sigma, St. Louis), endothelial cell growth factor 20 μg/ml (Organon Teknika Corp.), L-glutamine 2mM (Sigma, St. Louis), penicillin (80 units/ml), and streptomycin (80 μg/ml), a mixed population of endothelial cell colonies and other

adventitial cells were present. The endothelial cells originating from the microvessel explants were washed free of hematopoietic cells, but areas of fibroblast growth were occasionally noted. At this stage, selective metabolic labeling of endothelial cells with Dil-acetylated LDL (Debili, N., et al., "In Vitro Effects of Hematopoietic Growth Factors on the Proliferation Endoreplication and Maturation of Human Megakaryocytes," Blood, 77:2326 (1991), which is hereby incorporated by reference) was used to estimate the purity of endothelial cells within each well. Endothelial cells were further purified from wells with smallest amount of fibroblast contamination (endothelial cell to fibroblast ratio: greater than 1:1) by positive selection using Ulex europaeus 1 ("UEA1"). UEA1 lectin (Sigma) was covalently bound to Tosyl activated DYNABEADS® M-450 (Dynal, Great Neck, NY), by the method of Sternberg, E.P., "Mechanisms of Platelet Production," Blood Cells, 15:23-47 (1989) and Radely, J.M., "Megakaryocyte Maturation in Long-term Culture," Exp. Hematol., 19:1075 (1991), which are hereby incorporated by reference. The mixed population of cells was treated with 1 mM EDTA, and 0.05% collagenase, washed twice in HBSS, and then resuspended in HBSS + 5% FCS at a cell density of 5 x 10^5 cells/ml. The cells were incubated for 10 minutes at room temperature with Ulex coated beads (50 beads/endothelial cell). After incubation, the BMEC bound to UEA1-coated beads were washed five times by resuspending them in 10 ml of HBSS + 5% FCS and mixing by end-over-end rotation for one minute, followed by separation using a magnetic particle concentrator ("MPC") (Dynal, Great Neck, NY). The contaminating cells in the washes were plated for further identification. The endothelial cells were detached from the UEA1 beads by incubation in HBSS + 5% FCS containing 0.01M fucose (L-isomer) (Sigma) for 10 minutes at 4°C and the beads were removed with MPC. The pure BMEC collected in each wash were pooled, centrifuged,

resuspended in ECGM and plated on gelatin coated tissue culture dishes. BMEC monolayers isolated in this fashion can be passaged for 8-10 times. Early passage cells (Aizawa, S., et al., "Molecular Basis of the Recognition of Intravenously Transplanted Hemopoietic Cells by Bone Marrow," Proc. Natl. Acad. Sci. USA, 85:3180 (1988); Tavassoli, M., et al., "Homing Receptors for Hemopoietic Stem Cells are Lectins with Galacyosyl and Mannosys Specificities," Trans. Assoc. Am. Phys., 100:294 (1994); Tavassoli, M., "Localization of Megakaryocytes in the Bone Marrow," Blood Cells, 15:3 (1989) and Springer, T.A., "Adhesion Receptors of the Immune System," Nature, 346:425 (1990), which are hereby incorporated by reference) were used for experiments described below.

Since a few microvascular endothelial cells may escape through the filtration step during collagenase digestion, the flow through from the digested material can be washed and then treated with Ulex coated DYNABEADS® to remove endothelial cells, or, alternatively, digested material can be plated on gelatin coated plastic dishes. This allows for the attachment of endothelial cells and removal of nonadherent hematopoietic cells. Subsequently, after 7 to 10 days of growth, contaminating cells can be weeded out or Ulex coated DYNABEADS® can be used to isolate endothelial cells from contaminating stromal cells which are predominantly adventitial fibroblasts, adipocytes, and attached megakaryocytes, and monocytes.

Since fibroblasts reside in close association with the subluminal surface of microvessels, contamination with fibroblasts is the major impediment to the isolation of pure endothelial cell monolayers. The degree of fibroblast contamination varies for each isolation, and depends on the size of microvessels, the extent of collagenase digestion, and success of UEA1 affinity separation. Bulky, branched microvessels do not attach to gelatin or fibronectin coated

plastic dishes, as easily as small, fragmented microvessels. Monitoring of spicules during collagenase digestion ensures optimal digestion of microvessels. The ratio of endothelial cells to fibroblasts (optimal ratio of BMEC to fibroblasts of greater than 1:1) is critical for successful Ulex selection. Sternberg, E.P., "Mechanisms of Platelet Production," Blood Cells, 15:23-47 (1989), which is hereby incorporated by reference. Attempts to decrease fibroblast contamination can significantly increase the yield of endothelial cells and accelerate the growth of BMEC monolayers. Fibroblasts attach to plastic dishes far more avidly than endothelial cells, are insensitive to brief EDTA (0.5mM) treatment, and require longer collagenase digestion (greater than five minutes with 0.1% collagenase) to detach from plastic dishes. Thus, endothelial cell colonies grown from microvessel explants can selectively be detached from monolayers containing contaminating fibroblasts by incubation in 0.5 mM EDTA, and brief exposure (2-3 minutes) to 0.05% collagenase. This intervention, in addition to mechanical depletion of areas of heavy fibroblast growth before Ulex selection, decreases contamination with fibroblasts, and increases the yield of isolated endothelial cells. Substitution of D-valine for L-valine in the culture medium, which reportedly slows the growth of fibroblasts (Imai, T., et al., "Interleukin-6 Supports Human Megakaryocytic Proliferation and Differentiation in vitro," Blood, 78:1969 (1991), which is hereby incorporated by reference), did not increase the yield of BMEC.

Example 2 - Bone Marrow Mononuclear Cell Preparation

Bone marrow aspirates were obtained from normal subjects undergoing bone marrow harvest at MSKCC. Ten ml of bone marrow aspirate was drawn into syringes containing 50 units/ml of preservative free heparin, and passed through an

80 micron filter to remove stromal elements. The bone marrow was diluted with buffer A, layered over ficoll-hypaque (density 1.077), and centrifuged at 400xg for 20 minutes, and the mononuclear cells at the interface were collected, washed in buffer A, and counted. Plastic adherent cells were removed by incubating ficoll purified marrow cells (5 x 10^5 cells/ml) on costar plastic dishes at 37°C for 2 hours. The nonadherent cells were passed again through a 80 micron mesh to remove any cell clumps, and resuspended in IMDM medium (Sigma), 20% fetal calf serum, and monothioglycerol (10 ng/ml) at a density of 5 x 10^5 cells/ml. For adhesion studies with BMEC, the cells were resuspended in HBSS supplemented with calcium (2mM) and magnesium (2mM).

Example 3 - CD34+ Progenitor Cell Isolation

Low-density bone marrow mononuclear cells (less than 1.077 g/ml) were separated over Ficoll-Paque (Pharmacia; Upsala, Sweden). CD34+ cells were enriched using a commercially available cell separation system kit from Cell Pro, Inc. (Debili, N., et al., "Expression of CD34 and Platelet Glycoproteins During Human Megakaryocytic Differentiation," Blood, 80:3022 (1992), which is hereby incorporated by reference), washed twice with 1% bovine serum albumin ("BSA") in phosphate buffered saline, resuspended in 1% BSA to a concentration of 2 X 10^8 cells/ml, and incubated for 25 minutes with a biotinylated anti-CD34 IgM monoclonal antibody (12.8) at room temperature. The cells were washed with 1% BSA to remove unbound antibody, then resuspended at 2 x 10^8 cells/ml in 5% BSA and loaded onto an avidin column. The adsorbed CD34+ cells were released by manually squeezing the gel bed, resuspended in IMDM with 20% FCS, and counted on a Coulter counter.

Example 4 - Assays

Monoclonal antibodies to factor VIII/vWF (Dako), PECAM (Becton Dickinson), thrombospondin (11.4, Oncogene Science, Manhasset, NY), ICAM1 (IOL54, AMAC), VCAM (1G11, AMAC), α-actin (Dako), BPIb (SZ2, AMAC), GPIIb/IIIa (10E5, a gift from Dr. B. Coller, Stony Brook Medical Center), LFA1 (IOT16, AMAC), L-Selectin (Dreg-56, a gift from E. Butcher, Stanford University), CD38 (AMAC), HLA-DR (Coulter), CD34 (HPCA-1, Becton Dickinson or 11.1.6, Oncogene Science), were used for immunohistochemical and adhesion assays.

In vitro adhesion assay

Plastic nonadherent bone marrow mononuclear cells (5×10^5 cells/ml) were incubated with HUVEC (Bruno, B., et al., "Further Examination of the Effects of Recombinant Cytokines on the Proliferation of Human Megakaryocyte Progenitor Cells," Blood, 77:2339 (1991), which is hereby incorporated by reference) or BMEC monolayers in HBSS with calcium and magnesium in 6 well cluster plates at 37°C for one hour with gentle shaking. The nonadherent cells were removed and adherent cells were characterized by light microscopy (Wright/Giemsa staining) and immunohistochemistry using monoclonal antibodies against CD34 (HPCA-1), GP1b (SZ2), and GPIIb/IIIa (10E5). GPIb or GPIIb/IIIa positive cells were counted in the entire two six well cluster plates and scored as megakaryocytes, and small round CD34+ but Factor VIII/vWF negative cells were scored as CD34+ progenitor cells. Erythroid and myeloid progenitors were identified morphologically by standard Wright/Giemsa staining.

CD34+ progenitor cells purified by avidin-biotin immunoadsorption column (Cell Pro) (Debili, N., et al., "Expression of CD34 and Platelet Glycoproteins During Human

Megakaryocytic Differentiation," Blood, 80:3022 (1992), which is hereby incorporated by reference), (10 μl of 10^6 cells/ml), were added to washed resting or stimulated BMEC monolayers cultured on Terasaki (Nunc, Illinois) or 96 wells. Adhesion assays were performed for 30 minutes at 37°C in HBSS supplemented with magnesium (2mM) and calcium (2mM), and unbound cells were removed by three washes with HBSS/Ca/Mg. Adherent cells on endothelial surfaces were counted manually using an inverted phase-contrast microscope. For adhesion inhibition studies, monoclonal antibodies (10 μg/ml) to CD34 (HPCA-1 BD, or 11.1 Oncogene Science), VCAM (1G11), LFA1 (IOT16) were incubated with BMEC on ice for 20 minutes prior to the study. In the case of CD34 inhibition assays, the antibody to CD34 (10 μg/ml) was also added to the fluid phase during the adhesion study.

Immunohistochemical Techniques

BMEC monolayers and microvessel explants were fixed in 3% formalin in PBS for 30 minutes then quenched with 0.1 M glycine in PBS pH 7.4 for 10 to 15 minutes with Histochoice (Amresco, Solon, Ohio), and blocked with 1.5% horse serum. Monoclonal antibodies to Factor VIII/vWF, CD34 (HPCA-1), PECAM, thrombospondin, ICAM1, VCAM, α-actin, GPIb, GPIIb/IIIa, or L-Selectin, at different dilutions were incubated with fixed cells, for one hour. After washing with PBS, biotylinated anti-mouse or anti-rabbit immunoglobulin diluted 1:200 in PBS containing 1.5% horse serum was incubated with cells for 30 minutes at room temperature. Endogenous peroxidase was quenched with 0.3% hydrogen peroxide in PBS for 30 minutes. Endogenous alkaline phosphatase was quenched with levamisole 1.25 mM for 20 minutes. After 30 minutes of incubation with avidin labeled peroxidase or alkaline phosphatase, slides were rinsed and incubated with peroxidase substrate, amino-ethyl

carbazole (red stain), or alkaline phophatase substrate fast red (red stain) for 10-20 minutes. After a final rinse, the cells were counter-stained with 1% hematoxylin.

Samples of bone marrow aspirate were gently placed on polylysine coated glass slides, air dried, and fixed with Histochoice or acetone/alcohol. The samples were stained with a primary mouse monoclonal antibody to Factor VIII/vWF for one hour at room temperature or overnight at 4°C, washed three times with phosphate buffered saline ("PBS"), and counterstained with goat anti-mouse IgG1-FITC. Photographs were taken with a Nikon fluorescence microscope using Kodak Ektachrome 160T ASA color film (Kodak, Rochester, NY, USA). FITC was visualized using standard FITC excitation/emission filter combinations.

Example 5 - Metabolic Labeling with Dil-Ac-LDL

Near confluent monolayers of BMEC or mixed populations of endothelial cells and fibroblasts were incubated with 10 μg/ml of Dil-Ac-LDL (acetylated low-density lipoprotein labeled with dioctadecyl 1,3,3,3,-tetramethyl-indocarbocyanine perchlorate, Biomedical Technologies Inc., Stoughton, MA), for four hours at 37°C. Debili, N., "In Vitro Effects of Hematopoietic Growth Factors on the Proliferation Endoreplication and Maturation of Human Megakaryocytes," Blood, 77:2326 (1991), which is hereby incorporated by reference. The cells were washed with HBSS with calcium and magnesium for 10 minutes, and were examined with a Nikon epifluorescence microscope with phase contrast optics. Dil-Ac-LDL uptake was visualized using standard rhodamine excitation/emission filter combinations.

Example 6 - Immunofluorescent Flow Cytometry

BMEC adherent and nonadherent CD34+ cells washed twice in PBS, resuspended in HBSS with 1% BSA, were incubated with saturating doses of monoclonal antibodies to CD34, CD38, HLA-DR, LFA1, for 30 minutes at 4°C. After washing, cells were stained with saturating amounts of FITC or rhodamine-conjugated goat anti-mouse IgG F(ab)2(Coulter). Controls were isotype matched non-immune Ig's and FITC conjugated anti-mouse IgG F(ab)2(AMAC). Cell associated immunofluorescence was assayed by quantitative flow cytometry using a Coulter Profile II.

Example 7 - Electron Microscopy

BMEC monolayers were washed three times with HBSS containing Ca/Mg, then fixed in 2% paraformaldehyde, 0.5% gluteraldehyde, followed by 1% osmium tetroxide. After dehydration, the samples were embedded for electron microscopy. 60-70 nm (silver-grade) sections were cut using a diamond knife (Diatome, Ft. Washington Pa) on a Sorvall MT-5000 microtome (DuPont). Sections were stained with 0.1% lead citrate and examined using a JEOL-100CXII electron microscope at an accelerating voltage of 80 KV.

BMEC were obtained from explants of microvessels and were characterized with respect to morphology, expression of factor VIII/vWF, thrombospondin, PECAM, CD34, and acetylated LDL uptake. Figure 1A is a photomicrograph of an intact bone marrow spicule stained with FITC-labeled monoclonal antibody to factor VIII/vWF, demonstrating the relative frequency of endothelial cells in the bone marrow and their close association with other hematopoietic elements, particularly mature megakaryocytes. Figure 1B is a photomicrograph of a partially collagenase digested bone marrow spicule demonstrating the complex vascular network of

adherent to BMEC resting monolayers respectively. This represents a twenty to thirty fold enrichment and suggests adhesion mechanisms specific to these cell types. Megakaryocytes and CD34+ progenitor cells adhered less well to HUVEC monolayers. The remainder of the adherent cells were comprised of plasma cells, mature myeloid precursors, and lymphoid-appearing cells (Table 1).

To examine the mechanism(s) of adhesion in greater detail, bone marrow CD34+ progenitor cells isolated by an avidin-biotin column were incubated with BMEC monolayers at 37°C for one hour. Ten percent of the added CD34+ cells were adherent to resting BMEC monolayers. This adhesion was partially blocked by antibodies to CD34 (HPCA-1, 11.1), and was blocked with EDTA (1mM); antibodies to VCAM, ICAM, or LFA1 did not block binding (see Figure 5). The phenotype of the adherent CD34+ progenitor cells that were detached from endothelial cells by brief treatment (one minute) with 0.5 mM EDTA was examined by flow cytometry. This revealed a relative enrichment for $CD34^+CD38^-$ $HLA\text{-}DR^-$ cells (Table 2) consistent with a more pluripotent progenitor cell phenotype. Tayrien, G., et al, "Purification and Properties of a Megakaryocyte Stimulatory Factor Present in Both Serum Free Conditioned Medium of Human Embryonic Kidney Cells and in Thrombocytopenic Plasma," J. Biol. Chem., 262:3263 (1987); Jackson, C.J., et al., "Binding of Human Endothelium to Ulex Europaeus I-Coated Dynabeads: Application to the Isolation of Microvascular Endothelium," J. Cell. Sci., 96:257 (1990); and Holthofer, H., et al. "Ulex Europaeus I Lectin as a Marker for Vascular Endothelium in Human Tissues," Lab. Invest., 47:60 (1982), which is hereby incorporated by reference). In contrast to the results obtained with resting BMEC monolayers, IL-1β treatment resulted in increased adhesion of CD34+ progenitor cells that was divalent cation independent and not inhibited by antibody to CD34 (see Figure 6).

TABLE 2

Phenotype of Adherent CD34+ Cells to BMEC Monolayer

	TOTAL	ADHERENT	NONADHERENT
CD34	85 ± 5	87 ± 5	82 ± 4
HLA-DR	52 ± 9	34 ± 10	72 ± 6
CD38	81 ± 10	39 ± 12	81 ± 5
LFA1 (CD11A)	15 ± 6	47 ± 9	11 ± 2

Phenotype of adherent CD34+ cells to resting BMEC monolayers. CD34+ progenitor cells purified by biotin-avidin immunoadsorption column from bone marrow mononuclear cells, were incubated with resting BMEC monolayers at 37°C for one hour, and the adherent population of CD34+ cells were detached from BMEC monolayers by brief (1 minute) EDTA (0.5mM) treatment. The adherent and the nonadherent population of CD34+ cells were incubated with 10 μg/ml of FITC-labeled monoclonal antibodies to CD34 (HPCA-1), HLA-DR, CD38, and LFA1 and the percentage of positive cells were determined by flow cytometry. The values in the table represent the percent positive cells. As shown in this table, the adherent population of CD34+ progenitor cells are enriched for the more pluripotent phenotype $CD34^{+}HLA\text{-}DR^{-}CD38^{-}$.

During the last few years, microvascular endothelia from brain, retina, adrenal gland, hemangiomas, corpus luteum, lung, adipose tissue, and recently myocardium (Favoloro, E.J., et al., "Endothelial Cells and Normal Circulating Haemopoietic Cells Share a Number of Surface Antigens," Thromb. Haemost., 61:217 (1989); Simmons, J.P., et al., "CD34 Expression by Stromal Precursors in Normal Human Adult Bone Marrow," Blood, 78:2848 (1991); Simmons, D.L., et al., "Molecular Cloning of a cDNA Encoding CD34, a Sialomucin of Human Hematopoietic Stem Cells," J. Immunol., 148:267 (1992); Fina, L., et al., "Expression of the CD34

Gene in Vascular Endothelial Cells," Blood, 75:2417 (1990); Soligo, D., et al., "Identification of CD34+ Cells in Normal and Pathological Bone Marrow Biopsies by QBEND10 Monoclonal Antibody," Leukemia, 5:1026 (1991); Ramani, P., et al., "QBEND10, a New Monoclonal Antibody to Endothelium: Assessment of Diagnostic Utility in Paraffin Sections," Histopathology, 17:2237 (1990); Anthony, P.P., et al., "Endothelial Markers in Malignant Vascular Tumors of the Liver: Superiority of QBEND10 Over von Willebrand Factor and Ulex Europaeus Agglutinin-1," J. Clin. Pathol., 44:29 (1991); Delia, D., et al., "CD34 Expression is Regulated Reciprocally with Adhesion Molecules in Vascular Endothelial Cells in vitro," Blood, 81:1001, (1993); and Heimfeld, S., et al., "Rapid Enrichment for Peripheral Blood Stem Cells Using a Unique Biotin-Avidin Immunoaffinity Separation System," FASEB, 6:1729a (abstract), which is hereby incorporated by reference) have been isolated and characterized and the importance of microvascular endothelial cell proliferation in tumor biology is well established. Nelien, M.A., "Thrombin Receptor Expression in Normal and Atherosclerotic Human Arteries," J. Clin. Invest., 90:1614 (1992) and Metcalf, D., "Hematopoietic Regulators: Redundancy or Subtlety?," Blood, 82:3515 (1993), which are hereby incorporated by reference. There are fundamental differences in the microvasculature of different organs. Microvascular endothelia within each organ express unique types of adhesion molecules, also referred to as organ specific-ECAMs. Zucker-Franklin, D., et al., "Characterization of Glycoprotein IIb/IIIa Positive Cells in Human Umbilical Cord Blood: Their Potential Usefulness as Megakaryocyte Progenitors," Blood, 2:347 (1992) and Asch, A.S., "Isolation of the Thrombospondin Membrane Receptor," J. Clin. Invest., 79:1054 (1987), which are hereby incorporated by reference. Asch, A.S., et al., "Cellular Attachment to TSP: Cooperative Interactions Between Receptor

Systems," J. Biol. Invest., 266:19833 (1990) and Asch, A.S., et al., "Thrombospondin Sequence Motif (CSVTCG) is Responsible for CD36-Binding," Biochem. Biophys. Res. Commun., 182:1208 (1992), which are hereby incorporated by reference, have shown that the murine lung vasculature expresses a specific 90 kd endothelial cell adhesion molecule ("Lu-ECAM") that may be responsible for selective metastasis of melanoma cells to the lung. Also, a 165,000 kd vascular endothelial cell antigen called endosialin, which is expressed in tumor microvasculature but is not found in blood vessels of normal tissues, or in any of the other normal adult cell types tested *in vivo* has been identified. Asch, A.S., "Analysis of CD36 Binding Domains: Ligand Specificity Controlled by Dephosphorylation of an Ectodomain," Science, 262:1436-1440 (1993), which is hereby incorporated by reference. Given the complex network, and close association of BMEC with hematopoietic and other adventitial elements within the bone marrow, we hypothesize that BMEC is a unique type of endothelium that may regulate hematopoiesis by direct cellular contact and/or expression and secretion of specific cytokines.

BMEC are morphologically similar to other types of endothelial cells, such as HUVEC, bovine aortic, and brain microvascular endothelial cells. Although BMEC grow in a cobblestone manner, they are more spindle shaped. They stain positively for factor VIII/vWF, PECAM, CD34 (early passages), and thrombospondin, but do not stain with antibodies to α-actin, GPIIb/IIIa, GPIb, L-selectin, VCAM or ICAM. BMEC monolayers express higher levels of PECAM at areas of cell-cell contact. PECAM, which is a 140-Kd glycoprotein, is also expressed on monocytes, neutrophils, megakaryocytes, and a subset of CD4 cells and may function as a transmigratory bridge permitting the exit of mature cells such as neutrophils, lymphocytes, and monocytes out of bone marrow. Paul, S.R., et al., "Molecular Cloning of a

cDNA Encoding Interleukin 11, a Strong Cell-Derived Lymphopoietic and Hematopoietic Cytokin," Proc. Natl. Acad. Sci. USA, 87:7512 (1990) and Warren, M.K., "A New Culture and Quantitation System for Megakaryocyte Growth Using Cord Blood CD34+ Cells and the GPIIb/IIIa Marker," Exp. Hematol., 21:1473 (1993), which is hereby incorporated by reference.

The selective homing of transplanted CD34+ progenitor cells to the bone marrow is likely to involve expression of specific adhesion molecules yet to be identified. The movement of mature myeloid and erythroid precursors, and even CD34+ progenitor cells from the bone marrow microenvironment to the peripheral circulation, is a complex phenomenon that is tightly regulated. Regulation of expression of specific adhesion molecules by BMEC or mature hematopoietic elements may allow for selective exit of these mature cells while the immature precursors remain bound to adventitial cells within the marrow. CD34 antigen is a 110 kd glycosylated protein, which is expressed on pluripotent hematopoietic progenitor cells, as well as on other vascular endothelial cells such as HUVEC, capillaries of different tissues (Heidenriech, R., et al., "Organization of the Gene for Platelet Glycoprotein IIb," Biochemistry, 29:1232 (1990) and Gordon, M.Y., et al., "Compartmentalization of a Hematopoietic Growth Factor (GM-CSF) by Glycosaminoglycans in the Bone Marrow Microenvironment," Nature, 326:403 (1987), which are hereby incorporated by reference) and neoplastic tissues, such as angiosarcomas, Kaposi's sarcomas, and hepatic hemangioendotheliomas. Roberts, R., et al., "Heparan Sulphate Bound Growth Factors: A Mechanism for Stromal Cell Mediated Haemopoises," Nature, 332:376 (1988) and Jaffe, E.A., "Culture of Human Endothelial Cells Derived from Umbilical Veins: Identification by Morphologic and Immunologic Criteria," J. Clin. Invest., 52:2745 (1973), which are hereby incorporated by reference. However, endothelium from large veins, arteries, placental and

lymphatics are CD34 negative. Heidenriech, R., et al., "Organization of the Gene for Platelet Glycoprotein IIb," Biochemistry, 29:1232 (1990), which is hereby incorporated by reference. Our findings, along with recent observations that CD34 is concentrated on interdigitating endothelial membrane processes (Heidenriech, R., et al., "Organization of the Gene for Platelet Glycoprotein IIb," Biochemistry, 29:1232 (1990) and Roberts, R., et al., "Heparan Sulphate Bound Growth Factors: A Mechanisms for Stromal Cell Mediated Haemopoises," Nature, 332:376 (1988), which are hereby incorporated by reference) suggest that CD34 may function as an adhesion molecule that mediates the transit of peripheral progenitor cells to the bone marrow. Our immunohistochemical studies indicate that only first passage BMEC monolayers are positive for CD34 but do not express this glycoprotein with subsequent passages. Adhesion studies demonstrate that CD34+ progenitor cells display affinity for resting BMEC (passages 2-4) monolayers that is divalent cation dependent and is partially inhibited by anti-CD34 antibody. Previous reports have shown that CD34+ progenitor cell binding to fibroblasts are enhanced with cytokine stimulation and expression of VCAM and ICAM (Gordon, M.Y., et al., "Heparin Sulfate is Necessary for Adhesive Interactions Between Human Early Hemopoietic Progenitor Cells and the Extracellular Matrix of the Marrow Microenvironment," Leukemia, 2:804 (1988) and Long, M.W., et al., "Human Hematopoietic Stem Cell Adherence to Cytokine and Matrix Molecules," J. Clin. Invest., 90:251 (1992), which are hereby incorporated by reference) adhesion molecules. However, antibodies to VCAM and ICAM do not inhibit binding, suggesting that selective binding of bone marrow derived CD34+ progenitor cells to BMEC may be mediated by other adhesion molecules. Recently, vascular CD34 has been identified as a counter receptor for leukocyte L-selectin (S. Baumhueter, et al., "Binding of L Selectin to

the Vascular Sialomucin CD34," Science 262:15 (1993), which is hereby incorporated by reference), raising the possibility that a similar interaction might mediate progenitor cell adhesion to L-selectin expressed by endothelium. Our data, however, show that BMEC (resting or stimulated with IL-1β) do not express L-selectin. These data suggest CD34+ progenitor cell binding to BMEC may be mediated by CD34 interaction with a ligand expressed specifically by resting BMEC. Several studies have shown that homing of murine progenitor cells to bone marrow is regulated by a calcium-dependent C-peptide. Simmons, P.J., "Vascular Cell Adhesion Molecule-1 Expressed by Bone Marrow Stromal Cells Mediate the Binding of Hematopoietic Progenitor Cells," Blood, 80:388 (1992) and Long, M.W., et al., "Thrombospondin Functions as a Cytoadhesion Molecule for Human Hematopoietic Progenitor Cells," Blood, 75:2311 (1990), which are hereby incorporated by reference. Our adhesion studies indicate that CD34+ cell binding to unstimulated BMEC monolayers can be completely abrogated with EDTA, supporting the notion that a specific calcium-dependent adhesion molecule may be responsible for the homing of CD34+ progenitor cells to the bone marrow.

The CD34+ cells that are adherent to BMEC monolayers are enriched for the $CD34^{+}HLA\text{-}DR^{-}CD38^{-}$ phenotype, which is reported to be a more pluripotent cell type within the CD34+ progenitor cell population. Tayrien, G., et al, "Purification and Properties of a Megakaryocyte Stimulatory Factor Present in Both Serum Free Conditioned Medium of Human Embryonic Kidney Cells and in Thrombocytopenic Plasma," J. Biol. Chem., 262:3263 (1987); Jackson, C.J., et al., "Binding of Human Endothelium to Ulex Europaeus I-Coated Dynabeads: Application to the Isolation of Microvascular Endothelium," J. Cell. Sci., 96:257 (1990); and Holthofer, H., et al. "Ulex Europaeus I Lectin as a Marker for Vascular Endothelium in Human Tissues," Lab.

Invest., 47:60 (1982), which are hereby incorporated by reference. Furthermore, adherent CD34+ progenitor cells proliferated in coculture with BMEC, suggesting that regulation of hematopoiesis may be effected by direct contact or by cytokines derived from this endothelium.

The role of inflammatory mediators such as IL-1β in regulating hematopoiesis is incompletely understood. IL-1β is known to regulate adhesion mechanisms that govern the transit of inflammatory cells from the circulation by increasing the expression of ICAM and VCAM on the luminal surface of the endothelium, resulting in upregulation of monocyte and polymorphonuclear cell adhesion. Hynes, R.O., "Integrins: Versatility, Modulation, and Signaling in Cell Adhesion," Cell, 69:11 (1992) and Tavassoli, M., et al., "Molecular Basis of Homing Intravenously Transplanted Cells to the Marrow," Blood, 76:1059 (1990), which are hereby incorporated by reference. Our studies demonstrate a similar increase in CD34+ progenitor cell adhesion to IL-1β treated BMEC monolayers. In contrast to the adhesion observed to resting (untreated) monolayers, adhesion of CD34+ progenitor cells to IL-1β treated BMEC monolayers was divalent cation independent and not inhibited by antibodies to CD34, VCAM, or ICAM. The regulation of BMEC adhesion of progenitor cells by IL-1β raises the possibility that increased transit mediated by increase adherence of these circulating hematopoietic cells is one mechanism by which the bone marrow can respond to inflammatory states. That independent mechanisms for cell adhesion are exhibited by resting and IL-1β stimulated BMEC fits well with experimental data showing that CD34 mRNA expression by HUVEC monolayers is rapidly downregulated by IL-1β, gamma-interferon, and tumor necrosis factor. D. Delia, et al., "CD34 Expression is Regulated Reciprocally with Adhesion Molecules in Vascular Endothelial Cells In Vitro," Blood, 81:1001 (1993), which is hereby incorporated by reference.

The specific enrichment of megakaryocytes, together with their previously described close association with the subluminal surface of the sinusoidal endothelium (E.P. Sternberg, "Mechanisms of Platelet Production," Blood Cells 15:23-47 (1989) and M. Tavassoli, et al., "Localization of Megakaryocytes in the Bone Marrow," Blood Cells 15:3 (1989), which are hereby incorporated by reference) raises the possibility that the final stages of megakaryocyte maturation and platelet formation may be regulated by bone marrow endothelium. The adhesion of a large number of megakaryocytes to BMEC monolayers relative to HUVEC monolayers, was particularly striking. Furthermore, immunofluorescence studies of multiple, intact spicules, as shown in Figure 1A, demonstrate that mature, large polyploid megakaryocytes reside at the subluminal surface of BMEC. Whether the interaction of BMEC with megakaryocytes are critical for megakaryocytopoiesis or thrombopoiesis remains to be determined.

Example 8 - BMEC-Derived Cytokines Support Progenitor Cell Growth and Differentiation

Self renewing potential is characteristic of true stem cells and *ex vivo* expansion of CD34+ progenitors as measured by expansion assays are a measure of the proliferative potential of the cells as well as the *in vitro* environment. Over time in culture, the capacity of a starting population of CD34+ progenitors to form CFU's diminishes as an inverse function of differentiation and correlates positively with self renewal of a pluripotent component. The ability of BMEC to serve as a feeder layer for the proliferation of CD34+ progenitors as compared with bone marrow fibroblasts, human umbilical vein endothelial cells or medium containing kit-ligand, IL-3, IL-6, erythropoietin, and G-CSF, has been examined. BMEC support

ex vivo CD34+ proliferation as well as do bone marrow-derived fibroblasts and better than HUVEC or medium along (Figures 7A and 7B).

To define known cytokines produced by BMEC, post culture supernatant of BMEC and HUVEC monolayers was examined for its content of IL-3 IL-6, SCF, G-CSF, and GM-CSF (Table 3). The addition of conditioned medium obtained from BMEC increased progenitor cell expansion in agarose assays beyond that obtained with K36EG, raising the possibility that a novel cytokine or cytokines may be responsible for these effects.

TABLE 3

Quantification of Known Cytokines Elaborated by BMEC and HUVEC Monolayers

SAMPLE (MDC)*	Control Medium (pg/ml)	HUVEC (pg/ml)	BMEC (pg/ml)
IL-3 (10)	0	39 ± 12	32 ± 11
IL-6 (3.13)	0	105 ± 19	292 ± 21
Kit-ligand (10)	150 ± 32	1141 ± 89	3000 ± 478
G-CSF (10)	0	0	345 ± 32
GM-CSF (1.5)	0	26 ± 12	373 ± 36

* MDC = minimal detectable concentration

Example 9 - Coculture of CD34 Progenitor Cells with BMEC Conditioned Medium Results in Enrichment and Expansion of Megakaryocytic Precursors

Studies on *ex vivo* expansion of hematopoietic stem cells have shown that only a few percent of CD34+ progenitor cells differentiate into megakaryocytic committed precursors. Nelken, N.W., et al., "Thrombin Receptor Expression in Normal and Atherosclerotic Human Arteries," J.

Clin. Invest., 90:1614 (1992), which is hereby incorporated by reference. However, CD34+ progenitor cells can proliferate into megakaryocytes in the presence of optimal megakaryocytic growth conditions. Recent *ex vivo* expansion studies on CD34+ progenitor cells, using a combination of kit-ligand (20ng/ml), IL-3 (50ng/ml), EPO (8u/ml), and GM-CSF (100ng/ml) have shown a 600 to 700 fold expansion of GPIIb/IIIa positive cells. Despite this several fold expansion, the percentage of GPIIb/IIIa positive cells comprise only 2 to 5% of the overall proliferating hematopoietic cells. Radely, J.M., et al., "Megakaryocyte Maturation in Long-Term Culture," Exp. Hematol., 19:1075 (1991), which is hereby incorporated by reference. The presence of a Meg-CSF acting in concert with these cytokines has been postulated (Metcalf, D., "Hematopoietic Regulators: Redundancy of Subtlety?," Blood, 82:3515 (1993), which is hereby incorporated by reference). Preliminary data suggests that BMEC may be a source of this activity.

To investigate the role of BMEC conditioned medium in mediating the differentiation of megakaryocytic precursors, CD34+ progenitor cells were plated in the upper chamber of a 24-well transwell plate (Costar) separated from direct contact with either BMEC, or HUVEC, or bone marrow fibroblasts, grown as monolayers on the lower chamber of the transwell plates. CD34+ progenitor cells were obtained by an avidin-biotin column from peripheral blood mononuclear cells (Cell Pro). Aliquots of CD34+ cells undergoing expansion in the upper chamber of transwell plates were removed at one week intervals and the phenotype of the expanding cells were characterized by immunohistochemistry, Wright/Giemsa staining and electron microscopy (Figures 8A and 8B). The preliminary results show that by day 21, 15 $\pm$ 1.2% of expanding cells grown in the presence of BMEC monolayers stain positively for GPIIb/IIIa (Figure 9). The increase of the GPIIb/IIIa expressing population seen in the

presence of BMEC monolayers as compared to fibroblasts or HUVEC monolayers is particularly striking. The results of this experiment suggests that the cytokines present in BMEC conditioned medium may selectively promote lineage specific maturation of CD34+ megakaryocytic precursors.

In our preliminary studies, inhibition by anti-CD34 antibody was achieved by coincubation of progenitor cells with BMEC, thus we have not yet distinguished homotypic interactions between CD34 expressed on BMEC and progenitor cells from heterotypic interactions between CD34 and another ligand. However, we have also noted that CD34 expression in BMEC is undetectable by immunohistochemistry beyond passage 1. Therefore, homotypic interactions are an unlikely explanation for the observed CD34-mediated adhesion in resting BMEC. Further, since L-selectin has recently been identified as a counter-receptor for CD34, it is important to examine its possible contribution to these data. In preliminary studies, L-selectin is not expressed on resting or activated BMEC as measured by immunohistochemistry.

Example 10 - Isolation, Purification, and Cloning of Putative BMEC-derived Stem Cell Growth and Differentiation Factors

Expression Cloning

Direct biochemical approaches to isolation of the cytokine(s) responsible for the activity that is observed to be problematic in that the biologic endpoints of our *in vitro* systems (delta assay, long term culture, megakaryocyte development) are likely to be mediated by several factors acting in concert. Metcalf, D., "Hematopoietic Regulators: Redundancy or Subtlety?," Blood, 82:3515 (1993), which is hereby incorporated by reference. An attractive alternative to direct biochemical isolation is functional expression

cloning in COS cells as described recently for IL-11. Paul, S.R., et al., "Molecular Cloning of a cDNA Encoding Interleukin 11, A Strong Cell-Derived Lymphopoietic and Hematopoietic Cytokine," Proc. Natl. Acad. Sci. USA, 87:7512 (1990), which is hereby incorporated by reference. The cDNA expression library is prepared from 5-10 μg of poly(A)⁺RNA derived from early passage BMEC monolayers that were treated with IL 1-beta for 24 hours and ligated into the COS-1 expression vector pCDNA-amp (Invitrogen). Three hundred pools of this library, each comprising 300-500 individual clones, are transfected into COS-1 cells by DEAE=dextran DNA transfection with the addition of 0.1mM chloroquine. Culture supernatants from COS-1 cells will be harvested 48 and 72 hours after transfection and assayed for an increase in activity in the culture systems described over that observed in the presence of kit-ligand (20ng/ml), IL-3 (50ng/ml), IL-1 (20ng/ml), EPO (8u/ml), GM-CSF (100ng/ml), and possibly IL-11 (20ng/ml). Very recent data shows that IL-11 is expressed by IL 1-beta treated cells and not only by resting BMEC. Hirt plasmid DNA preparation followed by additional transfection cycles will be performed further to subdivide positive pools and assayed to isolate a single cDNA containing plasmid. Standard methods for cloning and sequencing will be employed. Recombinant expression of putative cytokines will result in protein of sufficient quantity for bioassay.

Two types of readout assays will be employed in these studies to facilitate screening of COS supernatants:

CD34 and GPIIb/IIIa expression will be followed using a microtiter ELISA based assay as recently described in Warren, M.K., et al., "A New Culture and Quantitation System for Megakaryocyte Growth Using Cord Blood CD34+ Cells and the GPIIb/IIIa Marker," Exp. Hematol., 21:1473 (1993), which is hereby incorporated by reference. Using this system, a relatively small number of CD34+ progenitor cells

(1000/well) can be used, and an increase in CD34 and GPIIb/IIIa expression over time can be followed. The culture conditions will be as previously described in our preliminary data except that kit-ligand, IL3, IL6, IL11, and GCSF will be present throughout to permit screening for only those pools that synergize with the known active cytokines elaborated by these cells. The results of these microassays will be confirmed using flow cytometric analysis, immunohistochemistry, and electron microscopy as needed.

Rapid screening may also be facilitiated by a IIb promotor-growth hormone construct. Heidenriech, R.R., et al., "Organization of the Gene for Platelet Glycoprotein IIb," Biochemistry, 29:1232 (1990), which is hereby incorporated by reference. This construct will be used to transfect the starting CD34+ progenitor population and assay for GH in the microtiter wells by ELISA. In this fashion, a non-destructive assay on well supernatants can be performed and the cells can continue to be cultured over time.

Direct Biochemical Approach to the Isolation of Putative Cytokines.

In planned experiments, the conditioned medium from the BMEC will be fractionated to isolate and purify the putative cytokine(s) to homogeneity. As with the expression cloning approach outlined, assays will be performed in the presence of kit--ligand (20ng/ml), IL-3 (50ng/ml), IL-1 (20ng/ml), EPO (8u/ml), GM-CSF (100ng/ml), and IL-11 (20ng/ml) so that novel rather than known BMEC-derived cytokines can be identified. Antibodies will be raised to the purified active material; also, direct amino acid sequencing will be performed by the Harvard microchemistry facility. A cDNA library derived from early passage BMEC will be screened with monospecific affinity purified antibody or with deduced oligonucleotides derived from

direct amino acid sequence analysis. Strategies for these approaches are described briefly below.

The bone marrow matrix is a rich source of cytokines that are bound reversibly by specific glycosaminoglycans. Gordon, M.Y., et al., "Compartmentalization of a Hematopoietic Growth Factor (GM-CSF) by Glycosaminoglycans in the Bone Marrow Microenvironment," Nature, 326:403 (1987) and Roberts, R., et al., "Heparin Sulphate Bound Growth Factors: A Mechanisms for Stromal Cell Mediated Haemopoiesis," Nature, 332:376 (1988), which are hereby incorporated by reference. The endothelial matrix is also capable of supporting some aspects of megakaryocyte development suggesting that the relevant cytokines elaborated by BMEC are likely to be localized in the matrix. Both PF4 and TGF-β are known heparin binding cytokine regulators of megakaryocyte development. Addition of heparin, fucoidin, chondroitin sulfate or heparin sulfate to our proliferation assays will allow us to identify specific polyanions that influence the activity of post culture supernatants and thus may be useful in establishing a separation method.

Both the eluate from heparin or other polyanion sepharose as well as the flow through will be added separately or in combination of Ulex-selected or GPIIb/IIIa selected megakaryocyte cultures, and the number of functional platelet-like particles produced will be quantified by flow cytometry. The fraction(s) with the highest amount of Meg-CSF or CD34+-sustaining activity will be fractionated by size and affinity chromatography using anion (Mono-Q) and cation (Mono-S) exchange resins. Fractions from each procedure or combinations of fractions will be bioassayed as described above and in Methods leading ultimately to purification.

An obvious concern would be if polyanion affinity chromatography failed to result in an active fraction.

Confounding results could be produced by coisolation with PF4 or TGF-beta, both of which inhibit platelet formation, for example, and might obscure the activity of an active thrombopoietin. Care will be taken to identify the fractions containing PF4 and TGF-beta, and, if these fractions are heterogeneous by SDS-PAGE, further purification by sizing chromatography will be performed. An alternative strategy to polyanion chromatography as the initial purification step will be ammonium sulfate fractionation. Post-culture supernatants will be separated into 0-20, 20-40, 40-60, and 60-100% fractions and dialyzed against phosphate buffered saline, pH 7.4. Active fractions will be further separated by anion exchange chromatography using Mono-Q and/or Mono-S FPLC and analyzed as described above.

Antibodies to the purified material will be raised and affinity purified and a BMEC cDNA prokaryotic expression library will be screened in order to clone and sequence the molecule. Alternatively, amino acid sequencing may provide an opportunity to use an oligonucleotide probe to screen the BMEC library.

Example 11 - Megakaryocyte maturation and platelet formation are mediated by BMEC derived humoral factors.

To examine the ability of BMEC to support megakaryocyte growth and platelet formation, Ulex or GPIIb/IIIa selected human megakaryocytes were incubated with BMEC monolayers at 37 C in medium containing IMDM, 10% fetal calf serum, adenosine 10^{-5}, theophylline 10^{-5}, and PGE1 $2.8X10^{-6}M$ (ATP medium, to prevent activation of platelets or megakaryocytes). Over 5 to 7 days, the megakaryocytes attach to BMEC monolyaers, enlarge, and form a single long pseudopod that eventually fragments into functional

platelet-like particles (Figures 11A, B, C). Electron microscopic examination of the platelet-like particles obtained from the supernatant of these cocultures demonstrated the presence of morphological features similar but not identical to intact human platelets (Figures 11E and F).

To examine the functional characteristics of these particles, flow cytometric analysis was performed, revealing a light scattering pattern that was consistent with platelet morphology, and positive baseline staining with antibodies to GPIIb/IIIa, and GPIb. Similar to functional platelets, these particles exhibited agonist dependent expression of GMP140 (P-selectin), an activation dependent platelet antigen (Figures 12A and B).

To determine if contact is necessary for the formation of platelet-like particles, Ulex or GPIIb/IIIa selected megakaryocytes were cultured in the presence of BMEC conditioned medium. Exposure to BMEC conditioned medium but not control medium derived from bone marrow fibroblasts or HUVEC monolayers, resulted in enlargement of megakaryocytes and the formation of randon pseudopods or proplatelets which formed functional platelet-like particles identical to those produced by contact between BMEC and megakaryocytes (Figure 11D). These platelet-like structures also have light scattering identical to normal human platelets, baseline staining with GPIIb/IIIa, GPIb, and agonist induced GMP140 expression (Figures 12C and D).

These are provocative findings that suggest that the *in vitro* formation of platelets might be possible and suggest that cytokine(s) elaborated by BMEC are responsible for platelet production by megakaryocytes. Cell contact appears to guide the directional formation of the proplatelet along and between endothelial cells.

Although the invention has been described in detail for the purpose of illustration, it is understood

that such detail is made solely for that purpose and variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention which is defined by the following claims.

WHAT IS CLAIMED:

1. A process for ex vivo expansion of hematopoietic progenitor cells comprising:

providing hematopoietic progenitor cells and

expanding the hematopoietic progenitor cells ex vivo with bone marrow endothelial cells or a cell line derived therefrom or with one or more cytokines therefrom to produce a cellular preparation with an increased number of hematopoietic progenitor cells.

2. A process according to claim 1, wherein said expanding is carried out with the bone marrow endothelial cells, the bone marrow endothelial cells supporting adhesion of megakaryocytes and bone marrow mononuclear cells.

3. A process according to claim 2, wherein the bone marrow mononuclear cells are selected from the group consisting of CD34+ progenitor cells, plasma cells, erythroid cells, and myeloid cells.

4. A process according to claim 3, wherein the megakaryocytes and the CD34+ progenitor cells bind to the bone marrow endothelial cells at a level of 20 to 30 times greater than the amount of megakaryocytes and the CD34+ progenitor cells in bone marrow in the presence of divalent cations.

5. A process according to claim 4, wherein the binding of the CD34+ progenitor cells to the bone marrow endothelial cells is inhibited by the CD34 antibodies and ethylenediaminetetraacetic acid.

6. A process according to claim 4, wherein binding of the CD34+ progenitor cells to the bone marrow

endothelial cells is enhanced by treatment of the bone marrow endothelial cells with interleukin-1β with such binding being divalent cation independent and not inhibited by ethylenediaminetetraacetic acid of CD34 antibodies.

7. A process according to claim 2, wherein the bone marrow endothelial cells show selective adhesion of $CD34^+HLA\text{-}DR^-CD38^-$ phenotype cells.

8. A process according to claim 1, wherein the bone marrow endothelial cells are prepared from a cDNA expression library from poly(A)$^+$RNA derived from early passage bone marrow endothelial cell monolayers.

9. A process according to claim 1, wherein the bone marrow endothelial cells have ATCC Accession No. CRL11666.

10. A process according to claim 1, wherein said expanding is carried out with one or more cytokines selected from the group consisting of one or more cytokines supporting progenitor cell proliferation, one or more cytokines supporting differentiation of pluripotent progenitor cells to megakaryocytes, one or more cytokines supporting platelet formation by megakaryocytes, and mixtures thereof.

11. A process according to claim 10, wherein the one or more cytokines are one or more cytokines supporting progenitor cell proliferation.

12. A process according to claim 11, wherein the bone marrow endothelial cells have an enhanced ability to cause progenitor cell proliferation compared to bone marrow fibroblasts and human umbilical cord vein endothelial cells.

13. A process according to claim 11, wherein the one or more cytokines support CD34+ pluripotent progenitor cell self-renewal.

14. A process according to claim 10, wherein the one or more cytokines are one or more cytokines supporting differentiation of pluripotent progenitor cells to megakaryocytes.

15. A process according to claim 10, wherein the one or more cytokines are one or more cytokines supporting platelet formation by megakaryocytes.

16. A process according to claim 1, wherein said expanding is carried out in an autologous serum.

17. A process for conducting autologous hematopoietic progenitor cell transplantation comprising:

obtaining hematopoietic progenitor cells from a patient prior to cytoreductive therapy;

expanding the hematopoietic progenitor cells ex vivo with bone marrow endothelial cells or a cell line derived therefrom or with one or more cytokines therefrom to produce a cellular preparation with an increased number of hematopoietic progenitor cells; and

administering the cellular preparation to the patient in conjunction with or after cytoreductive therapy.

18. A process according to claim 17, wherein said expanding is carried out with the bone marrow endothelial cells, the bone marrow endothelial cells support adhesion of megakaryocytes and bone marrow mononuclear cells.

19. A process according to claim 18, wherein the bone marrow mononuclear cells are selected from the group consisting of CD34+ progenitor cells, plasma cells, erythroid cells, and myeloid cells.

20. A process according to claim 19, wherein the megakaryocytes and the CD34+ progenitor cells bind to the bone marrow endothelial cells at a level of 20 to 30 times greater than the amount of megakaryocytes and the CD34+ progenitor cells in bone marrow in the presence of divalent cations.

21. A process according to claim 20, wherein the binding of the CD34+ progenitor cells to the bone marrow endothelial cells is inhibited by the CD34 antibodies and ethylenediaminetetraacetic acid.

22. A process according to claim 20, wherein binding of the CD34+ progenitor cells to the bone marrow endothelial cells is enhanced by treatment of the bone marrow endothelial cells with interleukin-1β with such binding being divalent cation independent and not inhibited by ethylenediaminetetraacetic acid of CD34 antibodies.

23. A process according to claim 18, wherein the bone marrow endothelial cells show selective adhesion of $CD34^+HLA\text{-}DR^-CD38^-$ phenotype cells.

24. A process according to claim 17, wherein the bone marrow endothelial cells are prepared from a cDNA expression library from poly(A)$^+$RNA derived from early bone marrow endothelial cell monolayers.

25. A process according to claim 17, wherein the bone marrow endothelial cells have ATCC Accession No. CRL11666.

26. A process according to claim 17, wherein said expanding is carried out with one or more cytokines selected from the group consisting of one or more cytokines supporting progenitor cell proliferation, one or more cytokines supporting differentiation of pluripotent

progenitor cells to megakaryocytes, one or more cytokines supporting platelet formation by megakaryocytes, and mixtures thereof.

27. A process according to claim 26, wherein the one or more cytokines are one or more cytokines supporting progenitor cell proliferation.

28. A process according to claim 27, wherein the bone marrow endothelial cells have an enhanced ability to cause progenitor cell proliferation compared to bone marrow fibroblasts and human umbilical cord vein endothelial cells.

29. A process according to claim 27, wherein the one or more cytokines support CD34+ pluripotent progenitor cell self-renewal.

30. A process according to claim 26, wherein the one or more cytokines are one or more cytokines supporting differentiation of pluripotent progenitor cells to megakaryocytes.

31. A process according to claim 26, wherein the one or more cytokines are one or more cytokines supporting platelet formation by megakaryocytes.

32. A process according to claim 17, wherein the hematopoietic cells are obtained from peripheral blood.

33. A process according to claim 17, wherein the hematopoietic cells are obtained from bone marrow.

34. A process according to claim 17, wherein said expanding is carried out in an autologous serum.

FIG.1A

FIG.1B

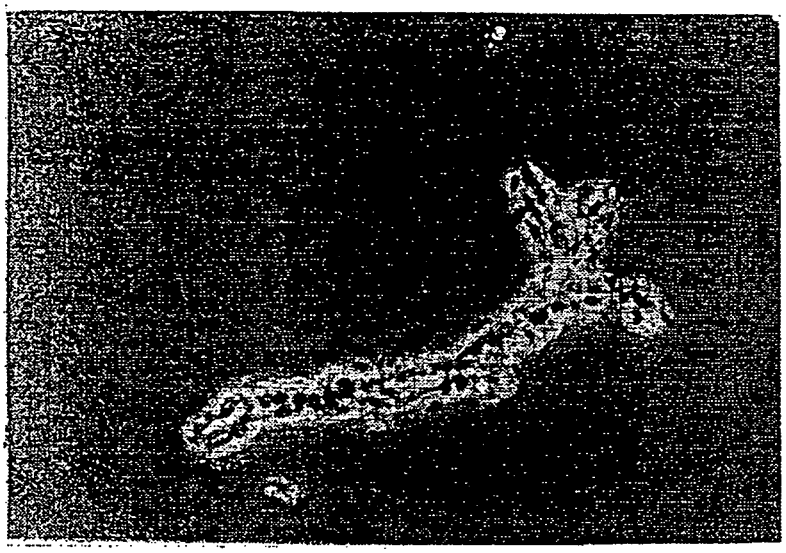

FIG. 2A

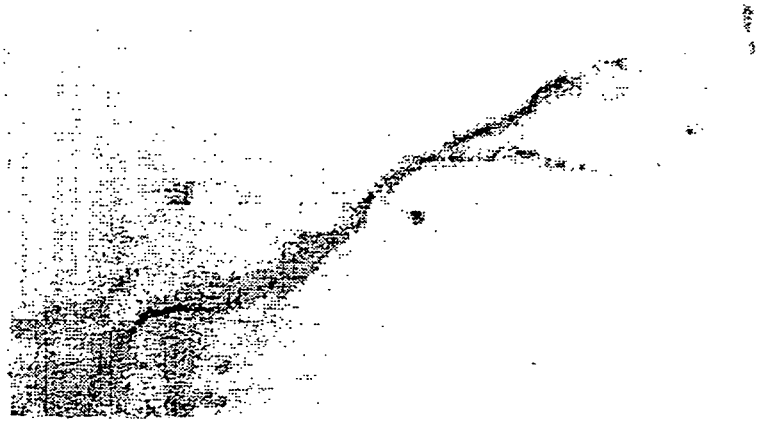

FIG. 2B

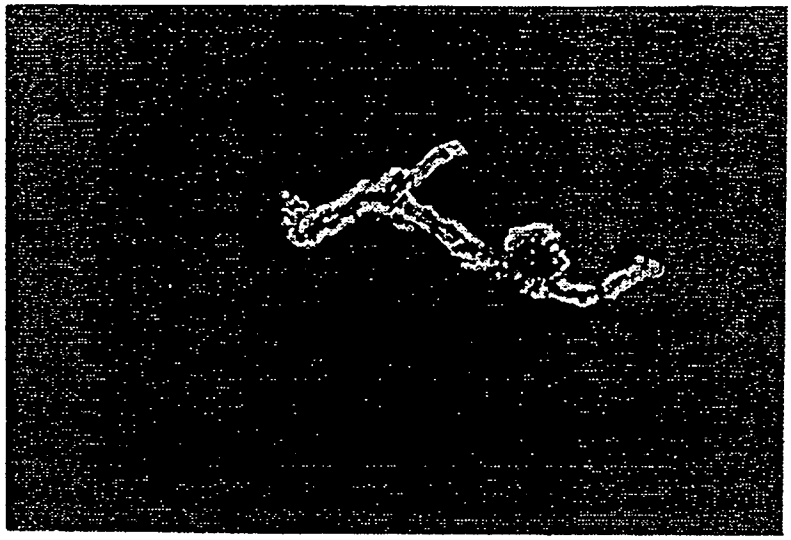

FIG. 2C

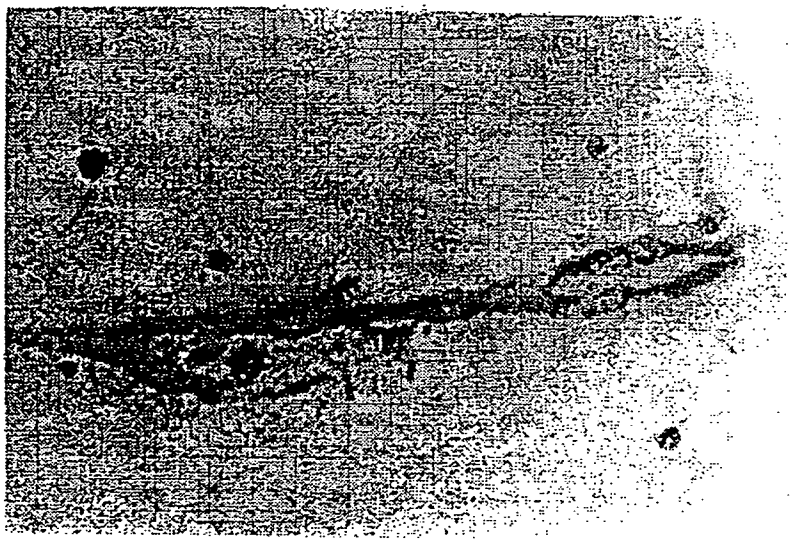

FIG. 2D

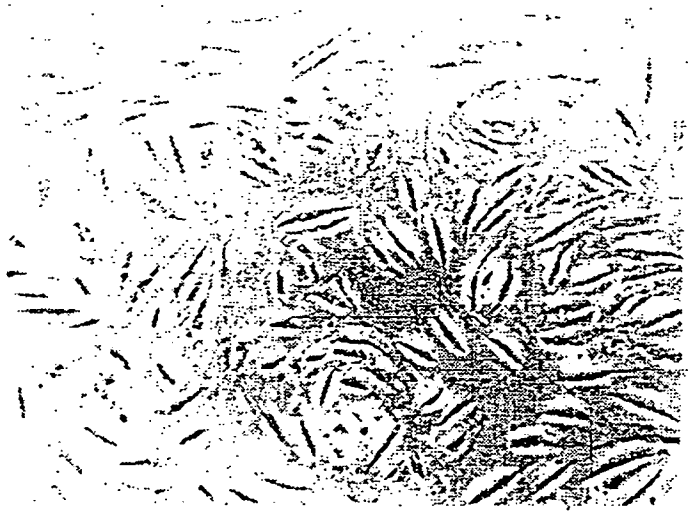

FIG.3A

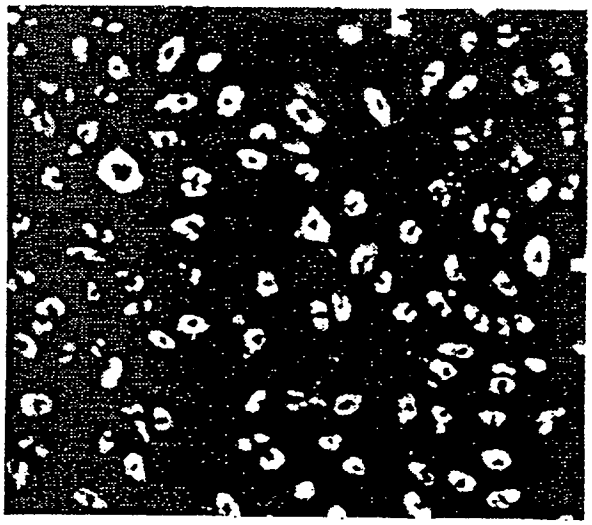

FIG.3B

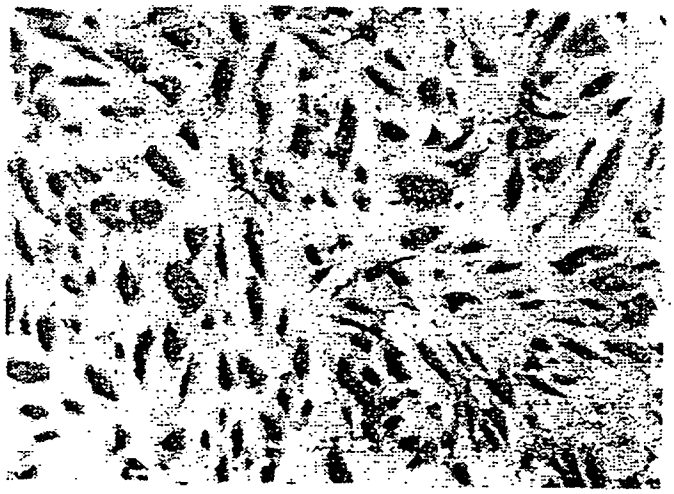

FIG.3C

FIG.3D

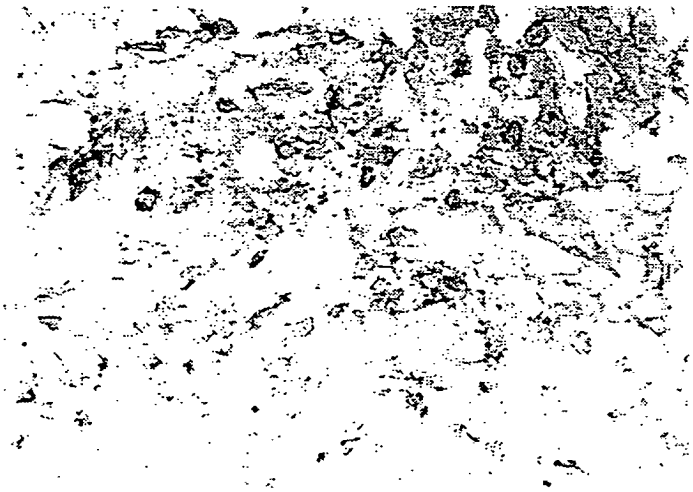

FIG.3E

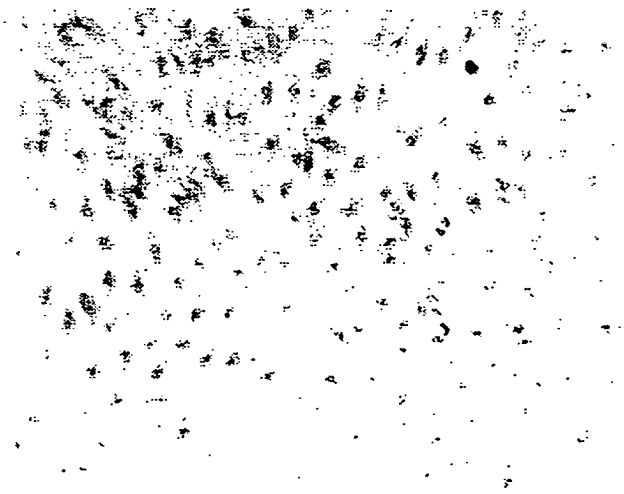

FIG.3F

FIG.4A

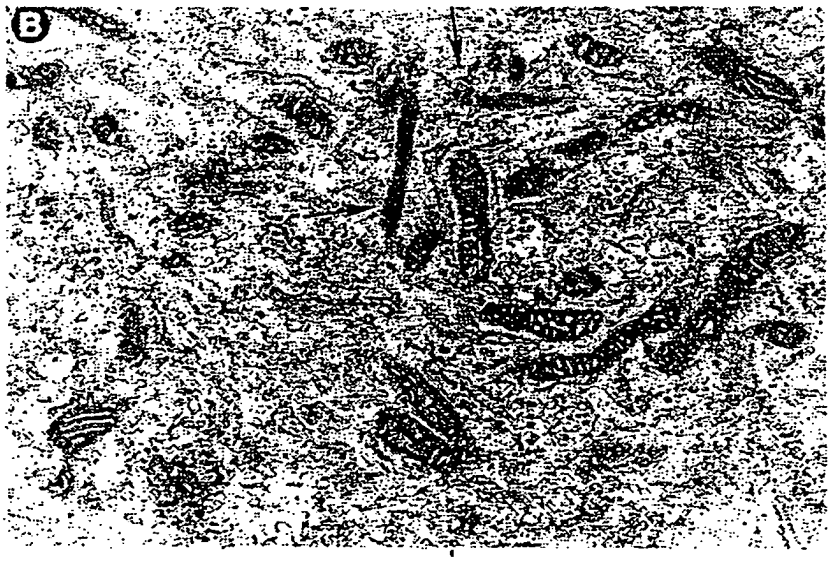

FIG.4B

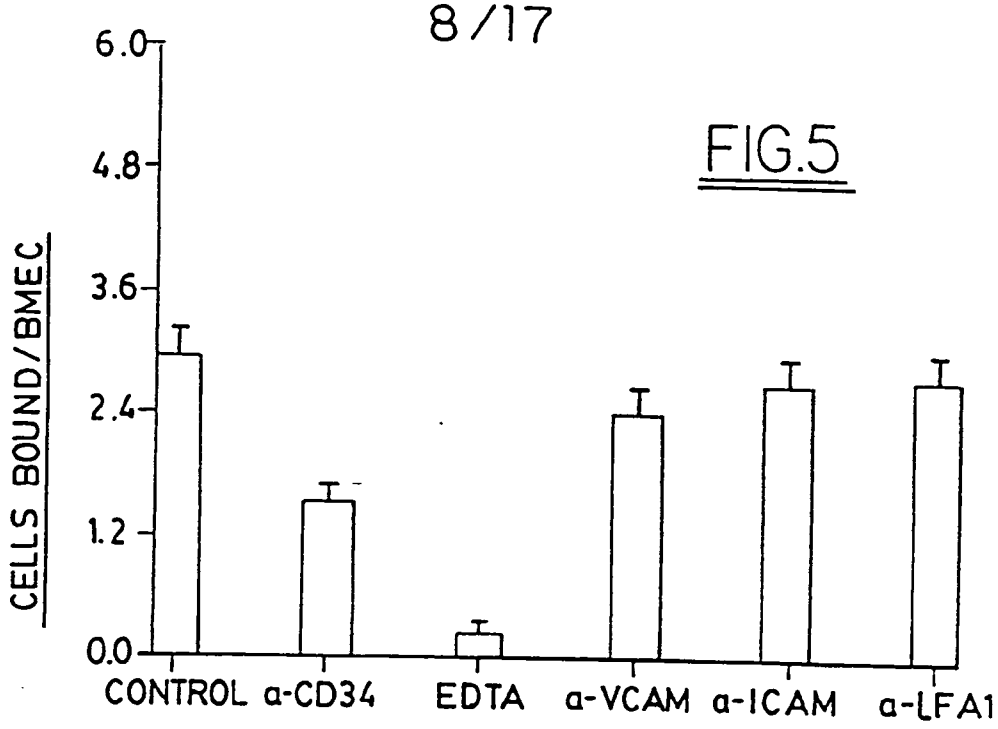
CELLS BOUND/BMEC
6.0
4.8
3.6
2.4
1.2
0.0
CONTROL
α-CD34
EDTA
α-VCAM
α-ICAM
α-LFA1

FIG.5

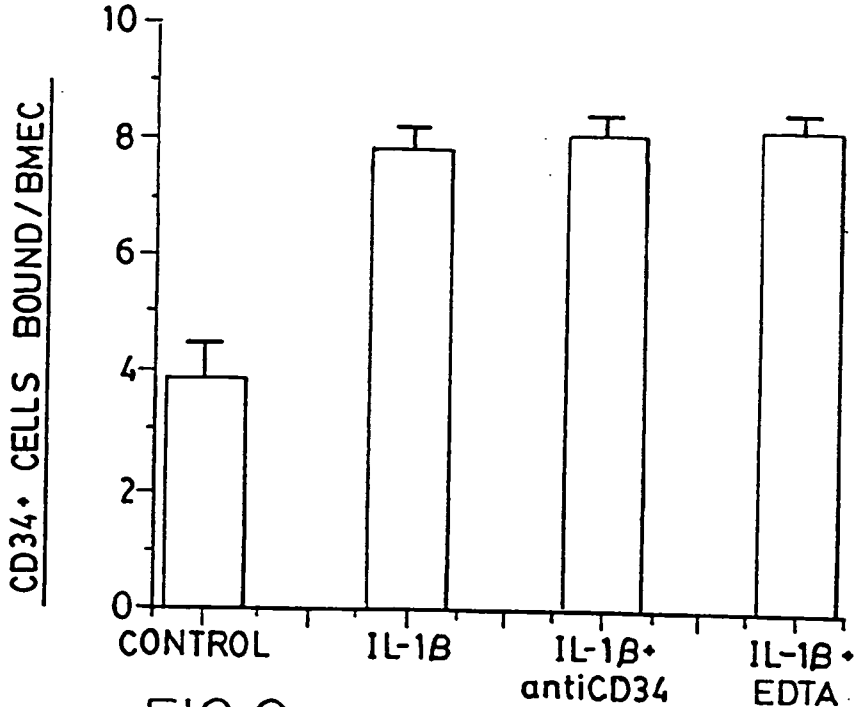
CD34+ CELLS BOUND/BMEC
10
8
6
4
2
0
CONTROL
IL-1β
IL-1β+ antiCD34
IL-1β+ EDTA

FIG.6

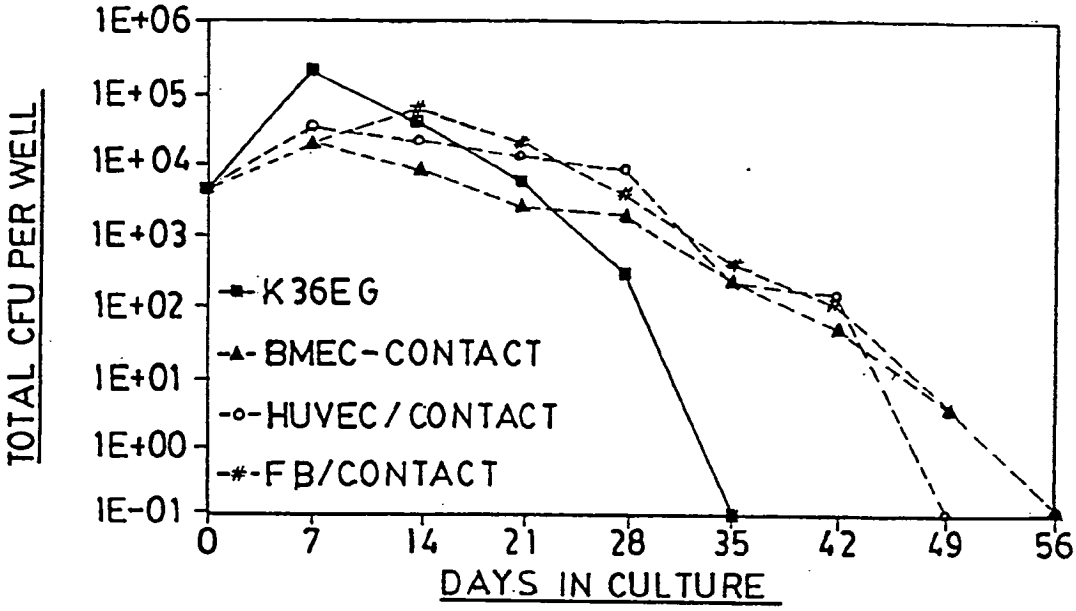
1E+06
1E+05
1E+04
1E+03
1E+02
1E+01
1E+00
1E-01
TOTAL CFU PER WELL
K36EG
BMEC-CONTACT
HUVEC/CONTACT
FB/CONTACT
0
7
14
21
28
35
42
49
56
DAYS IN CULTURE

FIG. 7A

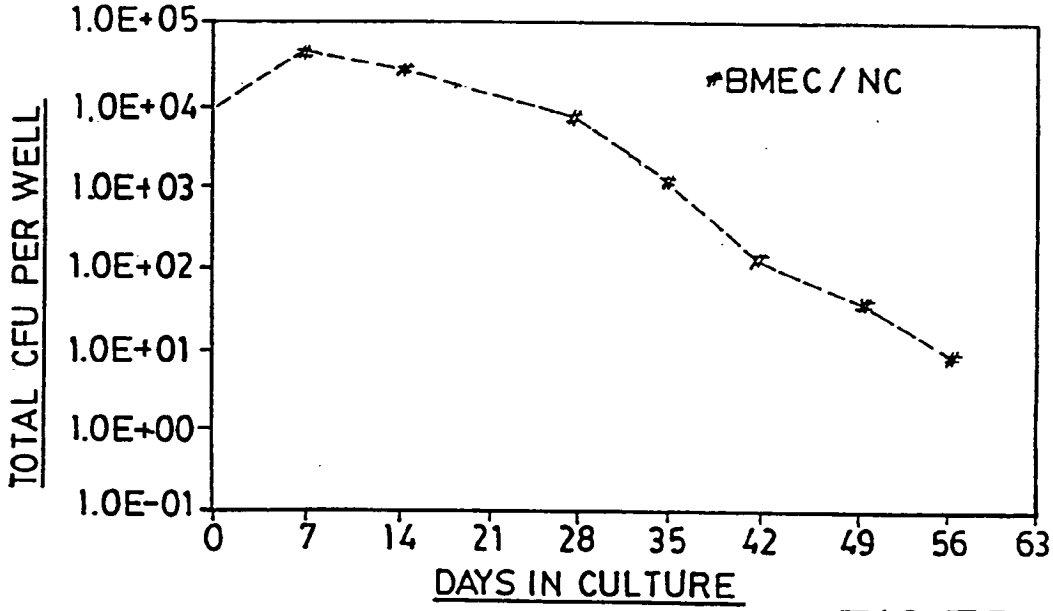
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
1.0E-01
TOTAL CFU PER WELL
BMEC/NC
0
7
14
21
28
35
42
49
56
63
DAYS IN CULTURE

FIG. 7B

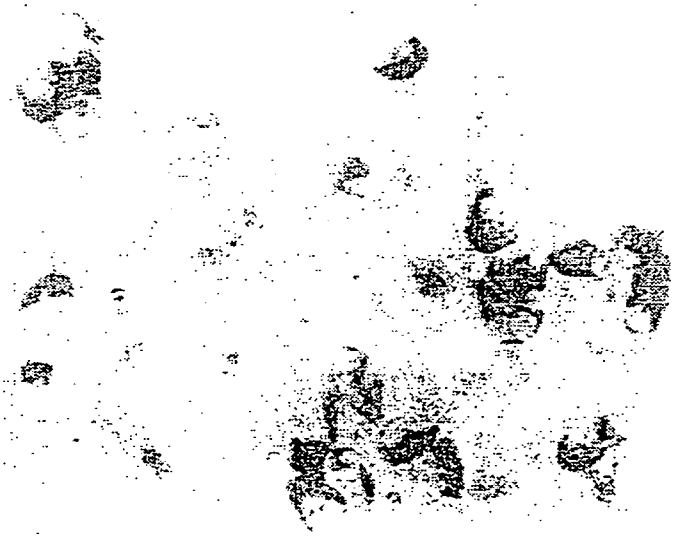

FIG.8A

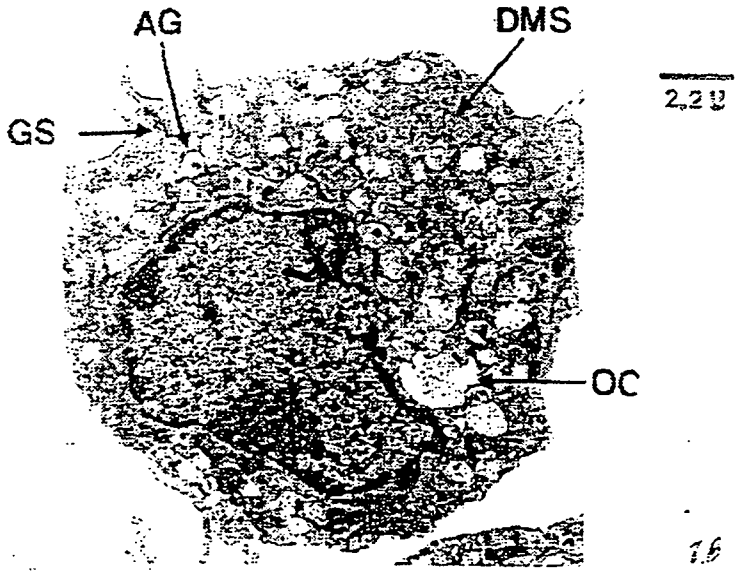

FIG.8B

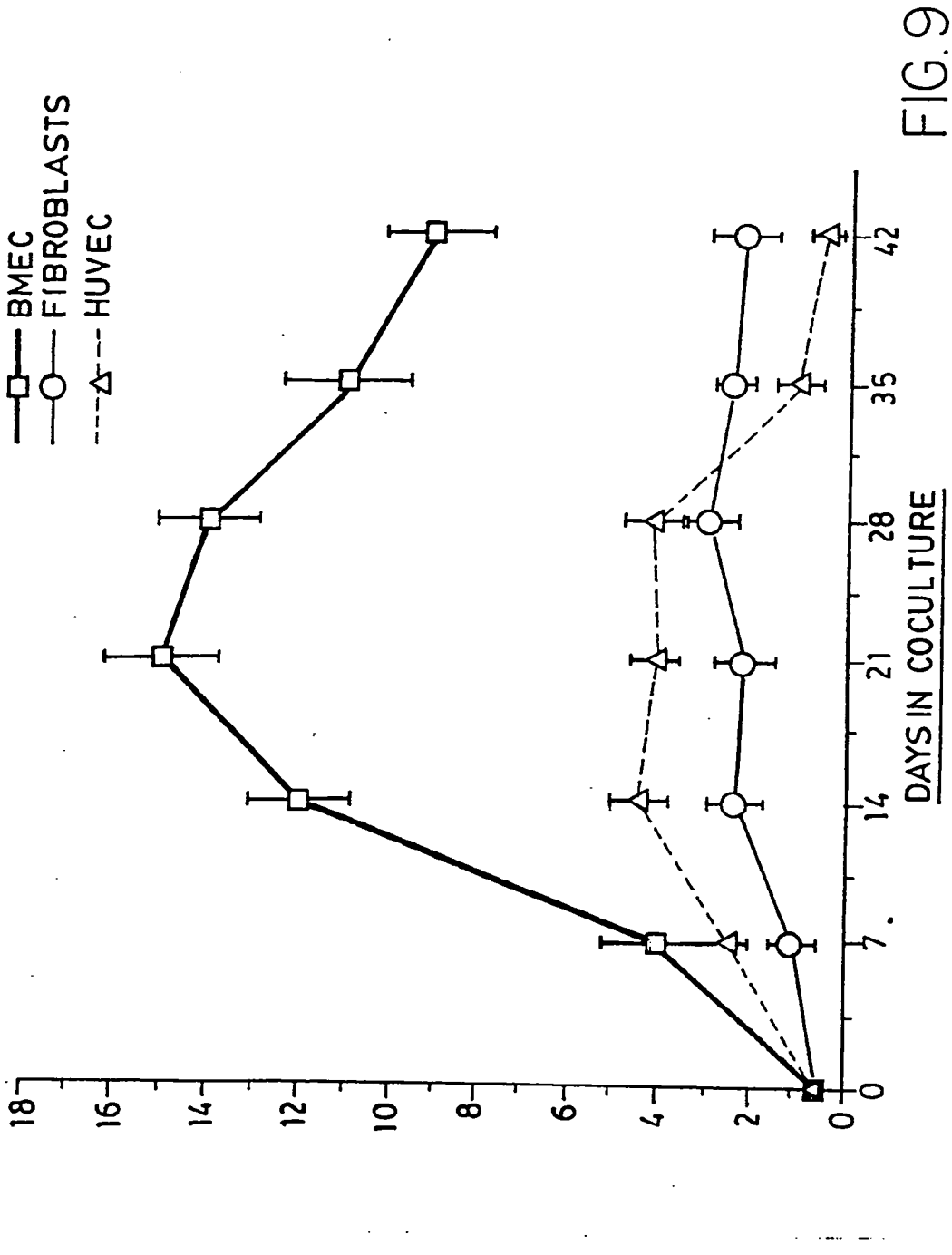
BMEC
FIBROBLASTS
HUVEC
18
16
14
12
10
8
6
4
2
0
0
7
14
21
28
35
42
DAYS IN COCULTURE

FIG. 9

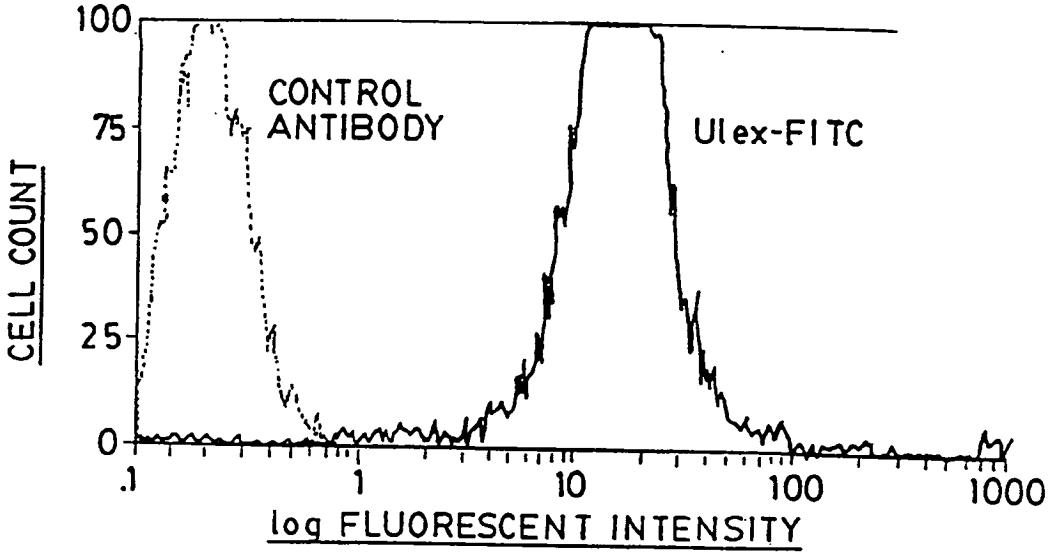

FIG. 10A

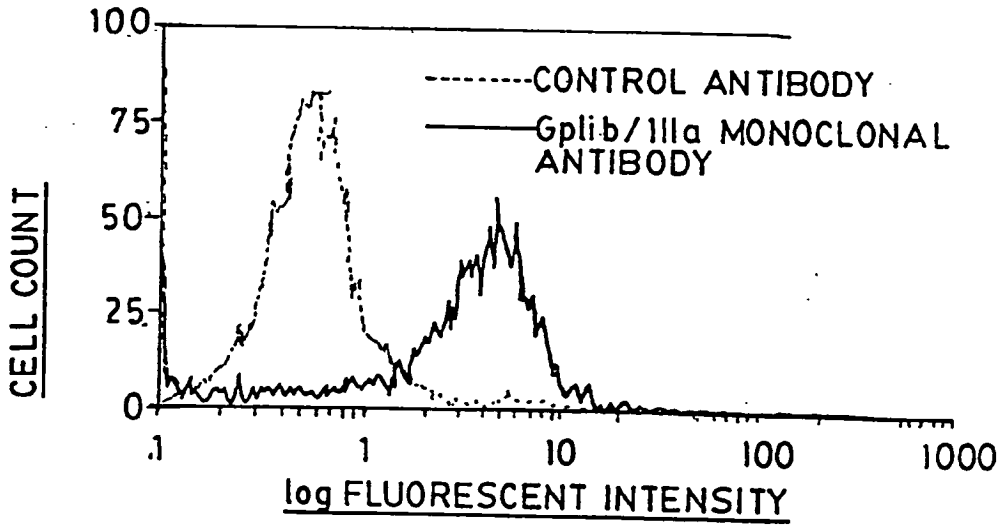

FIG. 10B

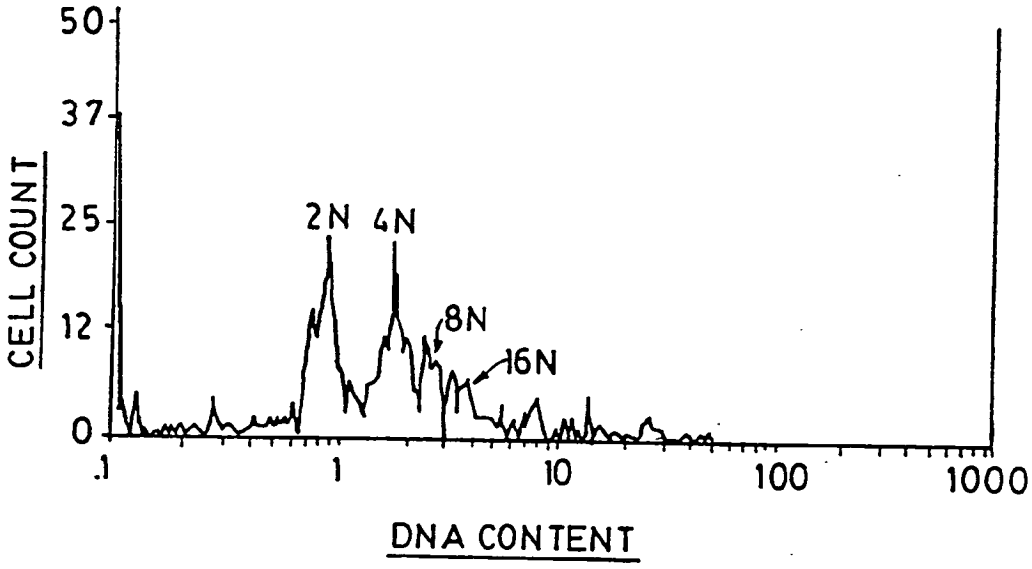

FIG. 10C

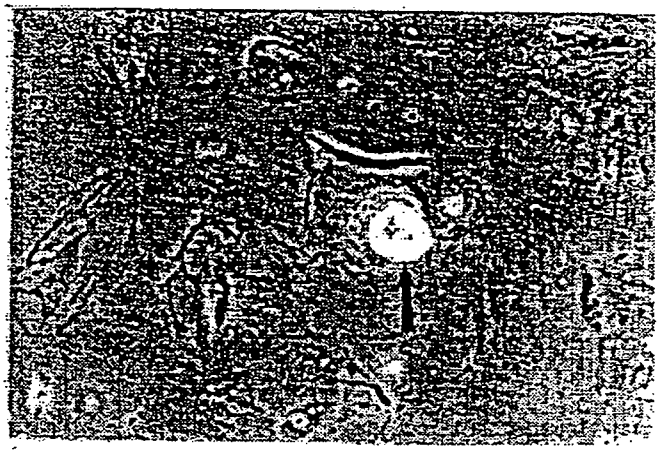

FIG.11A

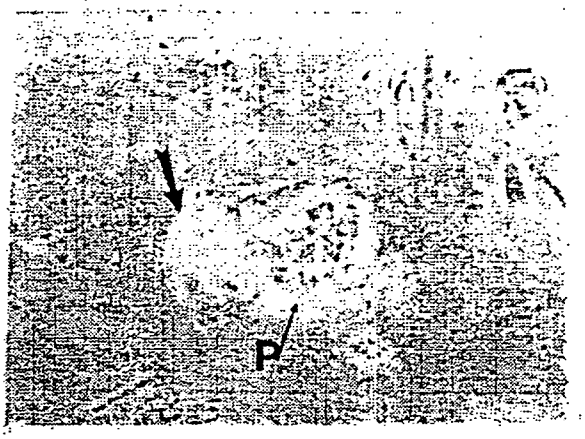

FIG.11B

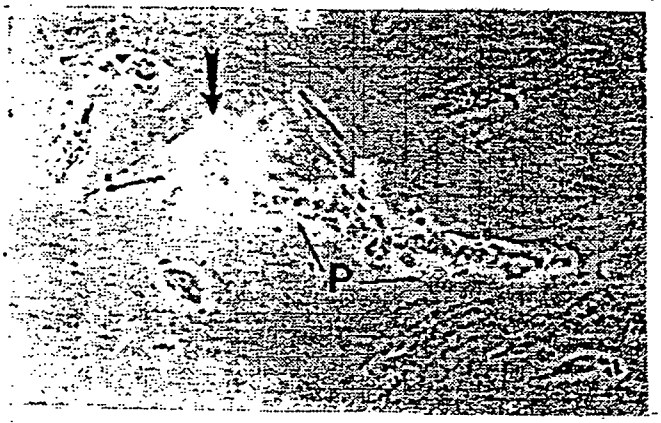

FIG.11C

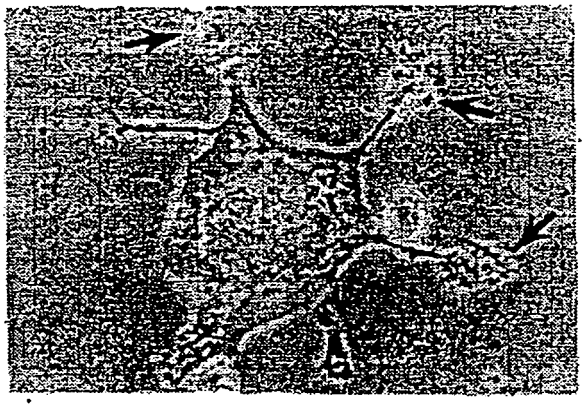

FIG.11D

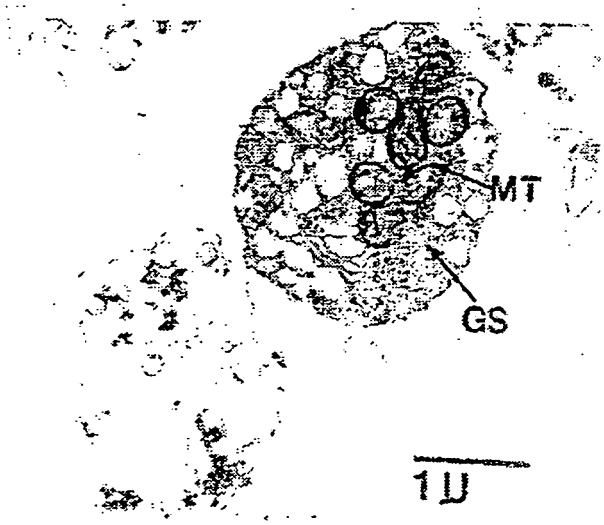

FIG.11E

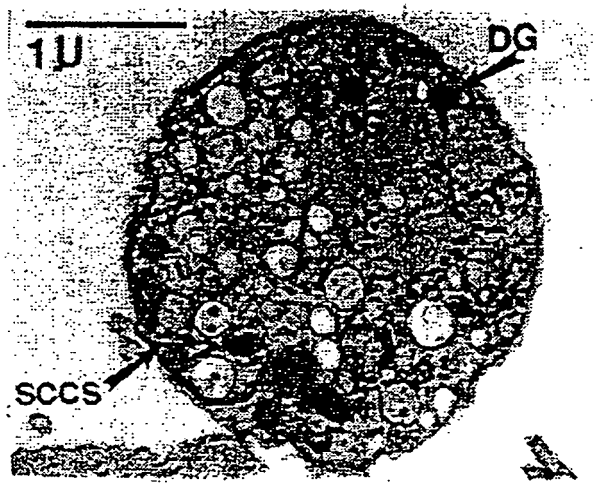

FIG.11F

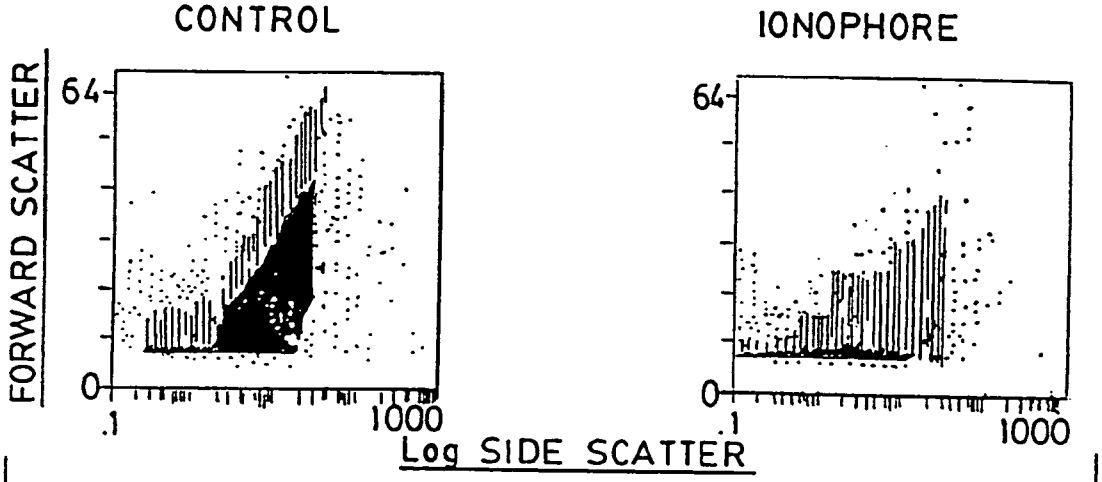

FIG.12A

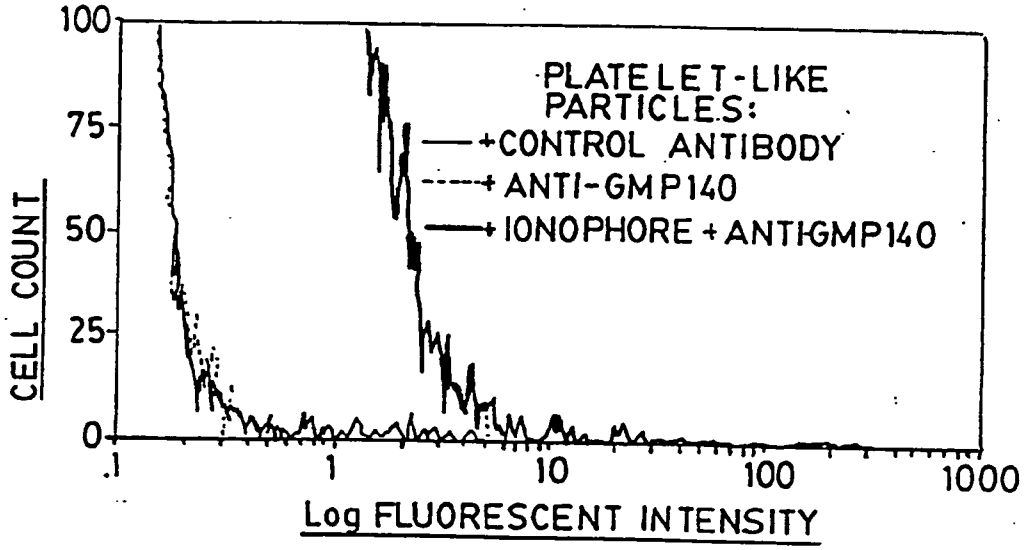

FIG.12B

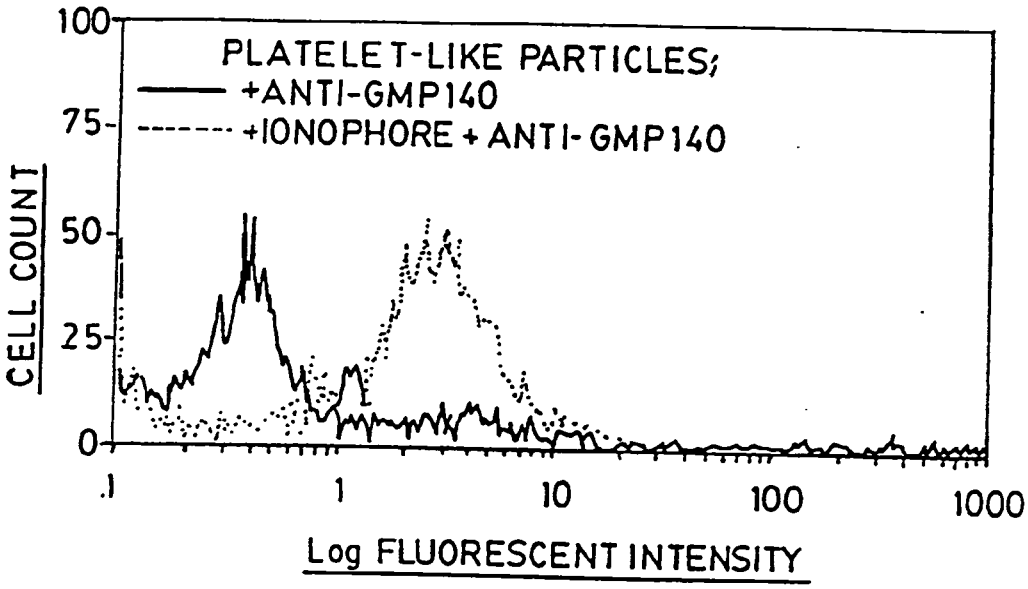
PLATELET-LIKE PARTICLES;
+ANTI-GMP140
+IONOPHORE + ANTI-GMP140
CELL COUNT
100
75
50
25
0
.1
1
10
100
1000
Log FLUORESCENT INTENSITY

FIG. 12C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/08031

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/00, 5/02; C12P 21/04; A01N 63/00; A61K 38/00.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.2, 240.25, 240.21, 240.3, 240.31, 70.1, 70.3, 70.5, 240.23, 240.243; 424/93.7, 93.1, 93.72, 93.73; 514/21.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog
search terms: hematopoietic, expand or expansion, bone marrow endothelial cells, cytokines

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,199,942 (GILLIS) 06 April 1993, see entire document.	1-34
Y	Dialog Abstract, issued 01 March 1993, "Role of Endothelial Cells in Blood Cell Production", abstract no. 0065793, see entire abstract.	1-34
P, Y	US, A, 5,409,825 (HOFFMAN ET AL.) 25 April 1995, see entire document.	1-34
T	US, A, 5,436,151 (MCGLAVE ET AL.) 25 July 1995, see entire document.	1-34
Y	Blood, Vol. 82, No. 10, issued 1994, Schweitzer et al., "ISOLATION OF HUMAN BONE MARROW ENDOTHELIAL CELLS", p. 22a, abstract #77.	1-9, 12, 16-25, 28, 32-34

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 SEPTEMBER 1995	24 OCT 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Susan M. Dadio Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/08031

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Blood, Vol 82, No. 10, issued 1994, Rafii et al., "HUMAN BONE MARROW MICROVASCULAR ENDOTHELIAL CELLS SUPPORT ADHESION OF HEMATOPOIETIC PROGENITOR CELLS", p. 22a, abstract # 76.	1-9, 12, 16-25, 28, and 32-34

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/08031

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/240.2, 240.25, 240.21, 240.3, 240.31, 70.1, 70.3, 70.5, 240.23, 240.243; 424/93.7, 93.1, 93.72, 93.73; 514/21.